FINAL REPORT

Characterization of Microbes Capable of Using Vinyl Chloride and Ethene as Sole Carbon and Energy Sources by Anaerobic Oxidation

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The background material for SERDP SON Number ERSON-07-04 notes that daughter products from reductive dechlorination of polychlorinated ethenes (including ethene and ethane) are often observed in situ, "… but the amounts detected are rarely sufficient to explain the disappearance of the chloroethenes, suggesting ethene may also be degraded, or that there may be alternative pathways for chloroethene degradation."

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ABBREVIATIONS AND ACRONYMS

AQDS 2,6-Anthraquinone Disulfonate
BES 2-Bromoethanesulfonic Acid

cDCE	*cis*-1,2-Dichloroethene
COD	Chemical Oxygen Demand
CSIA	Carbon Specific Isotope Analysis
DCM	Dichloromethane
DDI	Distilled, Deionized
dpm	Disintegrations Per Minute
EDTA	Ethylenediaminetetraacetic Acid
ETE	Ethene
ETA	Ethane
GC	Gas Chromatograph
GLF	Glucose, L-Asparagine, and Ferric Citrate
HPLC	High Performance Liquid Chromatograph
IC	Ion Chromatograph
MNA	Monitored Natural Attenuation
MSM	Mineral Salts Medium
PCE	Tetrachloroethene
SERDP	Strategic Environmental Research and Development Program
TCE	Trichloroethene
VC	Vinyl Chloride
VOC	Volatile Organic Compound

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EXECUTIVE SUMMARY

BACKGROUND

The background material for SERDP SON Number ERSON-07-04 notes that daughter products from reductive dechlorination of polychlorinated ethenes (including ethene and ethane) are often observed *in situ*, "… but the amounts detected are rarely sufficient to explain the disappearance of the chloroethenes, suggesting ethene may also be degraded, or that there may be alternative pathways for chloroethene degradation." For example, vinyl chloride (VC) may undergo direct oxidation to CO_2 or incomplete oxidation to acetate, which is further metabolized to CO_2 and CH_4; none of these products are discernible from other sources. Alternatively, it is possible that VC and ethene are consumed under microaerobic conditions, as a consequence of oxygen diffusing into groundwater at levels too low to detect and consumed at rates too high to accumulate. A number of microcosm studies have provided evidence in support of anaerobic oxidation of VC and ethene. However, it remains unknown what microbes might be performing these activities.

Considerable progress can be made towards understanding the anaerobic fate of VC and ethene if an isolate or highly enriched culture is obtained that uses these compounds as sole sources of carbon and energy. It may then become feasible to develop a gene probe to determine if VC or ethene oxidizers are present in field samples. If the phylogeny of the microbes responsible is not sufficiently unique to justify use of a 16S rDNA gene probe, it may still be possible to develop a gene probe based on a gene that is unique to anaerobic metabolism of VC or ethene. Development of such a tool would greatly improve the prospects for documenting natural attenuation when anaerobic VC or ethene bio-oxidation is occurring and for determining how widespread VC and ethene oxidizing microbes are. Obtaining isolates will make it possible to study the pathway for anaerobic VC or ethene metabolism.

OBJECTIVES

The overall objective of this research project was to culture and ultimately isolate and characterize microbes that are capable of using VC as a sole source of carbon and energy by anaerobic oxidation. The project scope was later expanded to include an evaluation of microbes capable of using ethene as a sole source of carbon and energy by anaerobic oxidation.

RESULTS

More than 700 microcosms were prepared with soil and groundwater samples from 11 sites, for the purpose of evaluating the occurrence of anaerobic oxidation of VC and ethene. The most important outcome from this effort was the development of a sulfate reducing enrichment culture that grows with ethene as its sole carbon and energy source. Ethene has previously been considered recalcitrant under anaerobic conditions. The evidence supporting ethene oxidation to CO_2 from this project includes the disappearance of considerable amounts of ethene without the detection of volatile organic products, such as methane or ethane, by gas chromatography, or of water soluble products like acetate by HPLC. Moreover, nearly all of the [^{14}C]ethene added to the enrichment culture was found in the $^{14}CO_2$ fraction after incubation with the culture, whereas only small amounts of $^{14}CO_2$ were detected in abiotic controls with most of the radioactivity

remaining as ethene. That sulfate was the electron acceptor was demonstrated by the near stoichiometric accumulation of sulfide in the samples amended with ethene, but not in controls lacking ethene. The production of sulfide and lack of known aerobic ethene oxidizers as major components of the 16S rRNA gene clone library argue against the possibility that ethene oxidation was due to small amounts of oxygen entering the microcosms and cultures.

The sulfate reducing enrichments were incubated in medium in which, other than trace amounts of vitamins, ethene served as the sole electron donor and organic carbon source, and sulfate was the only added electron acceptor other than CO_2. These cultures were transferred multiple times, a condition that requires growth of the biocatalyst. Ethene utilization rates increased over time in the cultures, also consistent with microbial growth. Moreover, microbes were readily apparent in cultures that had consumed ethene, and bacterial numbers, as estimated by qPCR, increased greatly relative to cultures lacking ethene or sulfate. Thus it is clear that the ethene/sulfate couple supported growth of microorganisms in these cultures.

The most numerous phylotype found in the 16S rRNA gene clone library, called MT6, is a member of the *Deltaproteobacteria,* most closely related to *Desulfovirga adipica* and several *Syntrophobacter* spp., organisms that carry out reactions with relatively low thermodynamic yields, and is somewhat more distantly related to *Desulfoglaeba alkanexedens*, a hydrocarbon utilizer. The MT6 16S rRNA gene is ≤91% identical with those from cultured organisms, and had ≤93% identity with the entire NCBI nr database, demonstrating how unique this phylotype is. While this distance precludes physiological conclusions based on phylotype, it is a reasonable candidate for an organism responsible for ethene oxidation and sulfate reduction.

In light of these and previous results, the possibility of ethene oxidation to CO_2 in sulfate reducing zones should be considered. If ethene is reduced to ethane *in situ*, the ethane can be readily detected by gas chromatography and contribute to mass balance determinations. However, if ethene is oxidized, it joins the large CO_2 pool and cannot be accounted for in the mass balance, leading to a mass balance deficit. Presently, there is no way to predict whether this reaction is occurring other than microcosm studies. It is possible that anaerobic ethene oxidation will have a stable isotope fractionation signature as does reduction to ethane. It is also possible that the unique bacterial phylotype we have found associated with this reaction can serve as a biomarker for it, but considerably more study will be needed to support or refute this hypothesis.

Definitive evidence in support of anaerobic bio-oxidation of VC was not found during this project. In a majority of the several hundred microcosms that were monitored (usually for one year or longer), VC was either recalcitrant or underwent reductive dechlorination to ethene. Consumption of VC without accumulation of ethene or ethane did occur in microcosms from one location at one of the sites. However, attempts to link the biodegradation of VC to a specific electron acceptor were unsuccessful, as were attempts to enrich for VC biodegradation by transfers to groundwater or medium. Furthermore, the possibility of oxygen serving as the electron acceptor (via diffusion through the septa) could not be completely ruled out. Consequently, a key need of SON Number ERSON-07-04 remains unfulfilled, i.e., identification of microbes capable of growing anaerobically on VC as the sole source of carbon and energy.

Several years prior to the start of this project, microcosms from an industrial site exhibited activity consistent with anaerobic bio-oxidation of VC. After consuming several repeat additions

of VC under presumptively anaerobic conditions, the rate of VC consumption slowed considerably and the microcosms were set aside. When this project started (six years after the original microcosms were established), an attempt was made to revive the VC biodegradation activity. All attempts to transfer material into defined medium or stimulation of anaerobic VC oxidation activity in these samples failed. The only activity recovered from the groundwater or original microcosms by culturing was the aerobic oxidization of VC. An aerobic VC oxidizer was subsequently isolated and identified as being related to previously described VC oxidizing *Mycobacterium* spp. This phylotype was also the most numerous in a bacterial 16S rRNA gene clone library, representing 38% of the total clones. The isolation of an aerobic *Mycobacterium* sp. strain from presumably anaerobic groundwater highlights the fact that groundwater contamination plumes are dynamic, and our understandings of the biogeochemical cycles within are not fully understood. Studies which track VC in shallow aquifers show VC mineralization under hypoxic conditions, though this is the first study to identify an organism that may be responsible for these *in situ* observations. Further studies must be performed to determine whether these strains of *Mycobacterium* spp. play an important role in *in situ* VC mineralization.

Despite microcosm evidence that anaerobic VC oxidation occurs within contaminated sites, the organisms involved have so far evaded enrichment and identification in the laboratory. At some of these sites, it is possible that oxygen is below the level of detection but high enough to allow for aerobic VC oxidation. Compound-Specific Isotope Analysis can be used to reduce uncertainty about the fate of biotransformed ethenes, but cannot fully explain the observed VC loss at contaminated sites. To accurately assess the flux of VC at sites where VC is observed to be decreasing without concomitant ethene formation, detection of known VC-oxidizing phylotypes like *Mycobacterium* spp. within a plume in comparison to non-contaminated groundwater could be indicative of aerobic oxidation of VC at levels of oxygen below detection, as would the ability to enrich aerobic VC oxidizers as we have in this study.

1.0 INTRODUCTION

The background material for SERDP SON Number ERSON-07-04 notes that daughter products from reductive dechlorination of polychlorinated ethenes (including ethene and ethane) are often observed *in situ*, "… but the amounts detected are rarely sufficient to explain the disappearance of the chloroethenes, suggesting ethene may also be degraded, or that there may be alternative pathways for chloroethene degradation."

There are several possible explanations for the lack of mass balances at field sites for chlorinated ethenes, including: 1) ethene produced from reductive dechlorination is oxidized to CO_2 as soon as it is produced; 2) VC is directly oxidized to CO_2; 3) VC is oxidized to acetate which is further metabolized to CO_2 and CH_4; or 4) oxygen diffuses into the system at below detection limits, and is continually consumed (Bradley, 2000; 2011; Bradley and Chapelle, 2000; Gossett, 2010). Production of ^{14}C-acetate, $^{14}CO_2$ and $^{14}CH_4$, from ^{14}C-labeled VC has been observed in microcosms (Bradley and Chapelle, 1999a; 2000). However, it remains unknown what microbe might be performing this metabolism (Bradley, 2000). Previous studies have reported that VC mineralization could be coupled to Fe(III) reduction and humic substances (Bradley and Chapelle, 1996; 1997; Bradley et al., 1998a). Aerobically, VC oxidation has been linked to *Mycobacterium* spp., *Nocardioides* spp. (Coleman et al., 2002) and *Pseudomonas* spp. (Verce et al., 2000), among others.

The initial focus of this study was anaerobic bio-oxidation of VC and was later extended to investigate anaerobic bio-oxidation of ethene. A summary of previous research on anaerobic bio-oxidation of VC and ethene follows.

1.1 BACKGROUND: ANAEROBIC MINERALIZATION OF VC

To date, no studies have cultured or identified organisms capable of anaerobic VC oxidation, even though it is thermodynamically feasible with various electron acceptors and despite the evidence from various field and microcosm studies. The conversion of VC to either acetate (Equation 1.1) or to bicarbonate (Equation 1.2) is energetically favorable under standard conditions. Using a terminal electron acceptor such as sulfate increases the favorability of the reaction (Equation 1.3) (Thauer et al., 1977).

$$C_2H_3Cl + 2H_2O \rightarrow CH_3COO^- + Cl^- + 2H^+ + H_2 \qquad \Delta G^{o\prime} = -166 \text{ kJ/rxn} \qquad (1.1)$$

$$C_2H_3Cl + 6H_2O \rightarrow 2HCO_3^- + Cl^- + 3H^+ + 5H_2 \qquad \Delta G^{o\prime} = -61.1 \text{ kJ/rxn} \qquad (1.2)$$

$$C_2H_3Cl + 1.25SO_4^{2-} + H_2O \rightarrow 2HCO_3^- + Cl^- + 1.25HS^- + 1.75H^+ \qquad \Delta G^{o\prime} = -251 \text{ kJ/rxn} \qquad (1.3)$$

The first report of VC bio-oxidation under anaerobic conditions was by Vogel and McCarty (1985). They fed [^{14}C]PCE to continuous flow fixed-film methanogenic columns and observed ^{14}C-labeled TCE, cDCE, VC and $^{14}CO_2$ as products. At the time, it was not yet recognized that VC could be further reduced to ethene, so they did not monitor for ethene. It was unclear, however, which of the compounds was undergoing oxidation. To evaluate this, they added unlabeled VC to dilute the concentration of labeled VC; this resulted in a reduction in the

concentration of $^{l4}CO_2$ exiting the column. On this basis, they concluded that PCE was stoichiometrically reduced to VC, which then underwent partial oxidation. This pathway was subsequently published in a widely cited review article (Vogel et al., 1987). Two years later Freedman and Gossett (1989) reported that VC can be further reduced to ethene. Most subsequent research on anaerobic dechlorination focused on reductive dechlorination, which led to important discoveries such as organohalide respiration of chlorinated ethenes by *Dehalococcoides* (Maymó-Gatell et al., 1997) and other genera. It was not until nearly one decade after the initial report by Vogel and McCarty (1985) that the topic of anaerobic oxidation of VC and ethene resurfaced in the literature, motivated in part by observations of poor mass balances for reductive dechlorination at numerous field sites. The following review is organized around the various groups that have reported on anaerobic oxidation since it was first proposed by Vogel and McCarty (1985).

1.1.1 Bradley, Chapelle, and Colleagues

Bradley, Chapelle and colleagues have produced an extensive number of publications that reported anaerobic mineralization of cDCE, VC and ethene. In one of their earlier studies, Bradley and Chapelle (1998) investigated the microbial mineralization of DCE isomers and VC under different electron accepting conditions. They documented the production of $^{14}CO_2$ from [^{14}C]DCE (a mixture of *cis*- and *trans*-) and [^{14}C]VC in microcosms constructed with aquifer and creek bed sediment, under aerobic, Fe(III)-reducing, sulfate-reducing, and methanogenic conditions. Although, they observed mineralization under all conditions, there were variations in the extent. The highest percentage recovery of $^{14}CO_2$ after a 50 day incubation period was observed under aerobic conditions: 64% in the aquifer microcosms and 100% in bed sediment microcosms. Approximately 21% recovery of $^{14}CO_2$ was reported for Fe(III)-reducing and sulfate-reducing conditions in the aquifer microcosms. A higher recovery occurred in the bed sediment microcosms; 70% under Fe(III)-reducing conditions and 52% under sulfate-reducing conditions. The least mineralization was observed in the methanogenic aquifer microcosms (9%, which was not significantly different from the killed controls). In the companion treatment with bed sediment, 39% mineralization was observed.

Bradley et al. (1998a) tested the hypothesis that the mineralization of VC was not coupled to methanogenesis. They showed that addition of humic acids to microcosms stimulated the rate of [^{14}C]VC mineralization and inhibited methanogenesis, suggesting that VC oxidation is not coupled to methanogenesis. AQDS, an analogue for humic acids, was provided as a terminal electron acceptor. They observed a 91% recovery of $^{14}CO_2$ in 50 days, with about an 80% reduction in methane production. Reduction of oxidized AQDS to reduced AQDS was demonstrated.

Bradley and Chapelle (1999a) also showed that methane production is a possible product of VC oxidation. Using radiometric detection headspace analysis, they observed that radiolabeled TCE and VC were biodegraded to $^{14}CH_4$. TCE was reductively dechlorinated to VC, which was then fermented to acetate (oxidative acetogenesis). When methanogenesis was not inhibited, the labeled acetate was fermented to $^{14}CH_4$ + $^{14}CO_2$ by acetotrophic methanogens. When methanogenesis was inhibited by addition of bromoethanesulfonate (BES), ^{14}C-acetate accumulated.

Taking into account their previous work, Bradley and Chapelle (2002) proposed several pathways for VC mineralization. One involves conversion of VC to acetate (oxidative acetogenesis), which can then be used as a substrate under all anaerobic electron accepting conditions. Fermentation of acetate to methane means that methane can be one of the ultimate endpoints of anaerobic chloroethene biodegradation. Another pathway involves direct oxidation of VC, although less is known about how this may occur. A third pathway proceeds by hydrogenolysis of VC to ethene, which may also be mineralized under sulfate-reducing conditions (Bradley and Chapelle, 2002).

1.1.2 Chang and Colleagues

Chang et al. (2003) investigated the possibility of anaerobic degradation of cDCE by microorganisms from a variety of sources, including drainage ditches, lakes, rivers, and ponds receiving landfill leachate. During a month of incubation in microcosms, 19 of the 80 samples tested were capable of degrading 10 µM of cDCE without accumulation of VC, ethene or ethane. A sample collected at a landfill area located in Nanji-do, Seoul, Korea, exhibited the highest extent of cDCE removal and it was used to create subcultures, to evaluate cDCE degradation under Fe(III) reducing, sulfate reducing, and methanogenic conditions. The greatest extent of cDCE consumption occurred under Fe(III) reducing conditions, followed by sulfate reducing conditions and methanogenic conditions. Subcultures were capable of degrading cDCE and VC in the presence and absence of Fe(III), suggesting the activity is not directly coupled to Fe(III) reduction. A major shortcoming of this study was the lack of evidence for mineralization, since ^{14}C-labeled cDCE and VC were not used.

1.1.3 Hata and Colleagues

A year after Chang and colleagues published their findings, Hata et al. (2003) replicated their work, using sediment samples collected from the same landfill in Nanji-do, Seoul, Korea. They also developed an anaerobic Fe(III) reducing enrichment culture capable of degrading cDCE and VC, without accumulation of ethene. Although the apparent mineralization of cDCE and VC occurred under Fe(III) reducing conditions, the activity was not linked to Fe(III) reduction, but rather depended on consumption of glucose. Hata et al. (2003) speculated that consumption of cDCE was an oxidative process, rather than reductive, since cDCE was degraded 3.5 times faster than VC, and VC and ethene did not accumulate. A major shortcoming of this study was the lack of direct evidence for mineralization, since ^{14}C-labeled cDCE and VC were not used.

In a subsequent study, Hata et al. (2004) isolated *Clostridium saccarobutylicum* strain DC1 from the anaerobic enrichment culture that grew on glucose. Following growth on glucose, strain DC1 consumed cDCE and VC, indicating the activity was cometabolic. Measurement of chloride ions was used to demonstrate stoichiometric degradation of cDCE. The significance of this finding must be viewed in light of the fact that the culture was grown on 10 g/L of glucose but consumed only ca. 40 µM cDCE and 20 µM VC; use of such a high dose of glucose would be impractical for *in situ* applications.

1.1.4 Freedman and Colleagues

Freedman and colleagues at Clemson University have investigated anaerobic oxidation of chloroethenes for several years. The results have been described in M.S. theses by Cline (2003), Pickens (2004), High (2008), Reid (2010), and Bakenne (2012). A summary of the work performed by High (2008), Reid (2010), and Bakenne (2012) is presented in this report. The previous work by Cline (2003) and Pickens (2004) provided a basis for this research; their work is summarized below.

An industrial site in Southern California was contaminated with TCE and dichloromethane (DCM). Field data indicates TCE has undergone reduction, with accumulation of cDCE and VC. However, the VC was much smaller than the cDCE plume and appeared to be stable despite there being minimal ethene present, which suggested anaerobic oxidation may be occurring. The microcosm studies by Cline (2003) and Pickens (2004) were designed to evaluate the possibility of anaerobic oxidation. Cline (2003) worked with samples near the source area, while Pickens (2004) evaluated samples taken further downgradient.

Cline (2003) found no evidence of anaerobic oxidation of cDCE, however, she did observe (presumptive) anaerobic oxidation of VC in two microcosms that were amended with [^{14}C]VC; 27-48% of the [^{14}C]VC was recovered as $^{14}CO_2$. Cline (2003) then transferred samples from the microcosms to fresh groundwater, to reduce the amount of soil present. Approximately 70% of the [^{14}C]VC added was recovered as $^{14}CO_2$. Repeated additions and consumptions of VC in the microcosms suggested utilization of VC as a growth substrate, however, the process was not conclusively linked to a specific terminal electron acceptor.

Pickens (2004) had similar results with the downgradient microcosms. One of the 18 bottles exhibited (presumptive) anaerobic oxidation of VC, i.e., consumption of VC without accumulation of ethene or ethane. This was confirmed by recovery of $^{14}CO_2$ from [^{14}C]VC. Transfer of samples to fresh groundwater also exhibited oxidative behavior. However, for unknown reasons, the rate of VC degradation slowed over time and ended before the terminal electron acceptor conditions could be determined.

Although the results from Cline (2003) and Pickens (2004) were promising in terms of demonstrating anaerobic oxidation of VC, an alternative explanation could not be ruled out: oxygen contamination. Although the microcosms and transfer cultures were prepared in an anaerobic chamber, it is conceivable that oxygen diffused in through the septa. Although resazurin was added as a redox indicator, both Cline (2003) and Pickens (2004) neglected to document the color of the microcosms during the period when VC was consumed. Also, neither measured the concentration of oxygen.

1.1.5 Gossett

The concern over oxygen contamination of microcosms that are presumed to be anaerobic is heightened by an elegant study by Gossett (2010). Using permeation tubes to slowly deliver oxygen to microcosms, Gossett (2010) demonstrated biodegradation of VC at oxygen levels that were too low to measure. This is a consequence of the very high affinity that aerobic bacteria have for oxygen when growing on VC as their sole carbon and energy source (Coleman et al.,

2002). Ethene was not tested; however, all of the bacteria that are known to grow aerobically on VC also grow on ethene, so it is reasonable to assume that they have the same level of affinity for oxygen when growing on ethene. The results from Gossett's study places a high burden of proof for any research seeking to demonstrate that bio-oxidation of VC or ethene occurs under truly anaerobic conditions.

1.2 BACKGROUND: ANAEROBIC MINERALIZATION OF ETHENE

Ethene is a non-toxic, gaseous, organic compound produced by plants as a phytohormone, and it and its precursor, aminocyclopropane-1-carboxylate can exude from roots into soils where the indigenous microbiota can convert it to ethene (Glick et al., 2007). Ethene can be produced by diverse soil bacteria (Primrose, 1979) and is found in trace amounts in marine sediments (Vogel et al., 1982). It is also a desired end product of anaerobic reductive dechlorination of chlorinated ethenes by *Dehalococcoides mccartyi* (Löffler et al., 2013; Maymó-Gatell et al., 1997) with vinyl chloride (VC) as its immediate precursor (Cupples et al., 2003; He et al., 2003).

Ethene and VC are readily oxidized to CO_2 by aerobes using monooxygenase reactions (Coleman et al., 2002; de Bont, 1976); however, anaerobic conditions in soils can lead to ethene accumulation (Goodlass and Smith, 1978). Little is known about the anaerobic metabolism of ethene. Bradley and Chapelle (2002) have provided the only evidence thus far for anaerobic oxidation of ethene, based on microcosms with lake-bed sediment from the Naval Weapons Industrial Reserve Plant in Dallas, Texas. [^{14}C]ethene was mineralized to $^{14}CO_2$. These results offered a potential explanation for the lack of ethene accumulation at sites where chlorinated ethenes undergo reductive dechlorination but the level of ethene is inadequate to explain complete dechlorination. Nevertheless, no information was provided about the microbes responsible; these studies have not led to active microcosms or cultures carrying out these reactions. Without such information, it will be difficult to develop tools that can be used for documenting *in situ* anaerobic mineralization of ethene.

The anaerobic oxidation of ethene to CO_2 is thermodynamically favorable only if coupled to an electron acceptor like sulfate; either directly in a single organism or via syntrophic couplings as shown in the following equations.

$$C_2H_4 + 6H_2O \rightarrow 2HCO_3^- + 2H^+ + 6H_2 \qquad \Delta G^{\circ\prime} = +88.7 \text{ kJ/mol} \qquad (1.4)$$

$$C_2H_4 + 1.5SO_4^{2-} \rightarrow 2HCO_3^- + 0.5H^+ + 1.5HS^- \qquad \Delta G^{\circ\prime} = -139.6 \text{ kJ/mol} \qquad (1.5)$$

Evidence has accrued for the slow anaerobic oxidation of alkanes, including ethane, propane, and butane (Kniemeyer et al., 2007), as well as for longer chain alkanes and alkenes (Grossi et al., 2008) by sulfate reducing bacteria, and organisms have been cultivated that mediate these oxidations. However, there is only scant evidence for anaerobic oxidation of ethene.

1.3 BACKGROUND: REDUCTION OF ETHENE TO ETHANE AND OTHER PATHWAYS

Another possible explanation for poor mass balances on chlorinated ethenes seen in the field is reduction of ethene to ethane (de Bruin et al., 1992; Koene-Cottaar and Schraa, 1998; Mundle et al., 2012), which is not as frequently monitored as ethene. Ethene reduction is a

thermodynamically favorable reaction with H_2 as the electron donor ($\Delta G^{o\prime}$ = -98.9 kJ/mole ethene).

Edwards and colleagues (Mundle et al., 2012) measured the $\delta^{13}C$ enrichment factor for reduction of ethene to ethane, using anaerobic microcosms developed at Clemson University with soil and groundwater from the Twin Lakes wetland at the Savannah River Site (SRS). In the process of enriching the microcosms for their ethene reduction capability, Johnson (2009) (a M.S. candidate in the laboratory of Dr. Edwards and Dr. Sherwood-Lollar) observed in several bottles that ethene was consumed without a commensurate increase in ethane. Instead, methane accumulated, and enrichment factors of -9.2±1.3l to -4.8±1.3l were measured, which is in agreement with published values for concerted breaking of a carbon double bond. Upon calculating the kinetic isotopic effect from the enrichment factors, their findings suggested the breakage of the carbon double bonds of ethene during the anaerobic biodegradation process, yielding methane and CO_2. Nevertheless, additional information about the culture has not yet been reported. Without a stable enrichment culture, it will be difficult to develop tools (including carbon specific isotope analysis) that can be used to document *in situ* anaerobic biodegradation of ethene. The findings of Johnson (2009) served as motivation for preparing new microcosms from SRS as part of the research for this project, with the intent of replicating the observation of ethene consumption without ethane formation.

1.4 KEY QUESTIONS

In spite of advances made in documenting the apparent occurrence of anaerobic VC and ethene mineralization in the field, more needs to be learned before it becomes practical to demonstrate the process as part of a monitored natural attenuation remedy or to enhance the process by addition of nutrients or even bioaugmentation. Among the key questions that remain unresolved are:

- Is anaerobic mineralization of VC and ethene a metabolic or cometabolic process? None of the studies reported thus far have provided much insight into this question. In a number of previous studies, the extent of anaerobic VC and mineralization reported has been low (e.g., below 25% of the [^{14}C]VC added). If metabolism was occurring, one might expect the rate and extent of mineralization to increase with time. We are not aware of any published attempts thus far that resulted in enrichment of, or isolation of the microbes responsible for anaerobic VC or ethene bio-oxidation.

- How can the process be documented *in situ*? Bio-oxidation of VC and ethene yields products that are not unique to chloroethenes, including CO_2, Cl^-, acetate, and methane. *In situ* levels of VC and ethene are not usually high enough to yield these products at concentrations readily discernible from background or from other contaminant sources. In the laboratory, ^{14}C-labeled VC and ethene have been used to demonstrate oxidation in microcosms. This is not a practical approach for routine application.

- How widespread is the process? All of the reports of VC bio-oxidation thus far have been made based on samples from only a few sites, including: Plattsburg Air Force Base near Plattsburg, New York (Bradley and Chapelle, 1996; Bradley et al., 1998b); Cecil Field Naval Air Station near Jacksonville, Florida (Bradley and Chapelle, 1996; 1997; Bradley

and Chapelle, 1998; Bradley and Chapelle, 1999a; Bradley and Chapelle, 1999b; Bradley and Chapelle, 2000; Bradley et al., 1998a); and the Naval Weapons Industrial Reserve Plant near Dallas, Texas (Bradley and Chapelle, 1999a; 2002). The laboratory results from the latter site were consistent with field evidence of VC attenuation based on a solute dispersion model and a mass balance assessment (Bradley et al., 1998b).

Considerable progress can be made towards answering these questions if an isolate or highly enriched culture is obtained that uses VC or ethene anaerobically as a sole source of carbon and energy. It may then become feasible to develop a gene probe to determine if VC or ethene oxidizers are present in field samples, in much the same manner that 16S rDNA gene probes are used to detect for *Dehalococcoides* spp. (Hendrickson et al., 2002). If the phylogeny of the microbes responsible is not sufficiently unique to justify use of a 16S rDNA gene probe, it may still be possible to develop a probe based on a gene that is unique to anaerobic metabolism of VC or ethene. Development of such a tool would greatly improve the prospects for documenting natural attenuation when anaerobic VC or ethene bio-oxidation is occurring and for determining how widespread VC and ethene oxidizing microbes are. Obtaining isolates will make it possible to study the pathway for anaerobic VC or ethene metabolism. In addition, it will be possible to determine if a microbe that grows anaerobically on VC or ethene as a sole substrate can also mineralize *cis*-dichloroethene (cDCE) (Bradley et al., 1998c).

1.5 OBJECTIVES

The overall objective of this research project was to culture and ultimately isolate and characterize microbes that are capable of using VC as a sole source of carbon and energy by anaerobic oxidation. The project scope was later expanded to include an evaluation of microbes capable of using ethene as a sole source of carbon and energy by anaerobic oxidation.

After monitoring hundreds of microcosms prepared with samples from numerous locations, the most significant finding from this project is the development of a sulfate-reducing enrichment culture that grows by anaerobic oxidation of ethene, in accordance with the stoichiometry predicted by equation 1.5.

2.0 MATERIALS AND METHODS

2.1 CHEMICALS

VC was obtained from Fluka or Sigma-Aldrich (>99.5%). TCE (Fisher Scientific, >99% purity), cDCE (TCI America, 99% purity), and DCM (Burdick and Jackson, >99.9% purity) were added to microcosms as water-saturated solutions. Ethene was obtained from Matheson (99.999%) or MG Industries (Chemically Pure, ≥99% purity). Ethane (99.995%) and methane (99.999%) were obtained from Matheson. High purity hydrogen (>99.99%) was obtained from National Specialty Gases. The quantities of gases used in experiments was calculated using the ideal gas law.

[^{14}C]VC (97.4% radiochemical purity, 1.4 mCi/mmol, dissolved in toluene with 0.1% p-methoxyphenol) was obtained from Perkin Elmer Life Sciences. [^{14}C]ethene was generated by adding [^{14}C]VC to an enrichment culture that reductively dechlorinates all of the chlorinated ethenes to ethene, using lactate as the electron donor (Eaddy, 2008). Then, samples of the headspace from bottles containing [^{14}C]ethene were added to the microcosms. For experiments with the sulfate-reducing culture developed by Fullerton and Zinder (section 3.12), a similar approach was used to generate additional [^{14}C]ethene. In this case, [^{14}C]cDCE was used instead of [^{14}C]VC. The [^{14}C]cDCE was purchased from Moravek Biochemicals (1.1 mCi/mmol) and a stock solution was prepared in DDI water. Approximately 10 mL of the stock solution was added to 1 L of the same enrichment culture described above, in a 1.18 L bottle, containing 0.18 L of headspace. Within three days, the [^{14}C]cDCE was converted to [^{14}C]ethene, about 57% of which was in the headspace of the bottle. Samples of headspace from the "stock" bottle were added to Balch tubes in two batches. For the first batch, 0.5 mL was added per tube, containing ca. 74,000 disintegrations per minute (dpm) of [^{14}C]ethene. For the second batch, it was necessary to add 3.0 mL per tube (due to diffusive losses of ethene from the "stock" bottle), containing ca. 38,000 dpm of [^{14}C]ethene.

An amorphic Fe(III) solution was made by adding 8 M NaOH to a 40 mM solution of $FeCl_3$ until the pH reached 7. The solution was then allowed to settle in the beaker, followed by subsequent centrifugation (Evolution RC, Sorvall®) for 15 min at 10,000 rpm (in 150 mL centrifuge tubes) and rinsing with DDI water (three times) to remove the majority of the chloride, giving an Fe(III) solution of approximately 200 mM (Lovley and Phillips, 1986; 1988). An amorphic manganese(IV) solution was made by slowly adding equal parts of a 30 mM $MnCl_2$ solution to a 20 mM $KMnO_4$ solution, while being stirred with a magnetic stir bar (Lovley and Phillips, 1986). The solution was then allowed to settle in the beaker before being centrifuged (Evolution RC, Sorvall®) for 15 min at 10,000 rpm (in 150 mL centrifuge tubes). It was subsequently triple rinsed with DDI water in order to remove the majority of the chloride, providing approximately 15 mM Mn(IV) as poorly crystalline MnO_2.

EDTA-Fe(III) (13% purity Iron) was obtained from J.T. Baker. Anthraquinone-2,6-disulfonic acid (AQDS), disodium salt (>99% purity) was obtained from Pfaltz and Bauer. Sodium sulfate (99.8% purity) was obtained from Fisher Scientific. All other chemicals used were of reagent grade. Sodium nitrate (99.0%) was obtained from Sigma. Resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide) (dye content approximately 80%) was obtained from Sigma-

Aldrich. Ethylenedinitrilotetraacetic (EDTA) acid, disodium salt was obtained from EMD (ACS grade). Technical grade yeast extract was obtained from Difco. Ferric citrate (purified) was obtained from Spectrum. L-asparagine monohydrate (98+%) was obtained from Alfa Aesar. D-glucose (ACS grade) was obtained from Mallincokrodt. Copper(II) oxide (ACS grade) was obtained from Alfa Aesar.

All other chemicals used were of reagent grade or an equivalent purity.

2.2 MEDIA

A number of the serum bottle microcosms (described in section 2.3.2) were prepared with various types of media rather than groundwater, and in other cases samples from microcosms were transferred to media. Seven types of media were used, depending on the desired treatment or enrichment culture. The components of each medium are in Table 2.1. A brief description of each medium follows. The amount of terminal electron acceptor shown in Table 2.1 is based on the amount of VC or ethene initially added; in some cases, more was added at a later time, as will be described in the Results.

For treatments that used oxygen and/or nitrate as the terminal electron acceptor, the phosphate-buffered media described by Hartmans et al. (1992) was used, with the following modification. Sodium nitrate was added as needed to a concentration of 2.1 mg N/L, providing an electron accepting capacity of 6.07 mg/L of chemical oxygen demand (COD).

For treatments that were designed to enrich for iron-reducing conditions, the bicarbonate and phosphate-buffered media described by Lovely and Phillips (1988) was used. Two types were prepared: Type #1 and Type #2. Type #1 was prepared identically to Lovely and Phillips (1988). For type #2, two modifications were made: The concentration of $MgSO_4 \cdot 7H_2O$ was decreased, to lower the amount of sulfate and thereby reduce the chances of developing sulfate reducing conditions; and the amount of $MgCl_2 \cdot 6H_2O$ was increased to maintain the same concentration of magnesium. For both types of iron-reducing media, treatments received equimolar amounts of Fe either as Fe(III) or as Fe(III)-EDTA. Acetate was added as the primary electron donor. Yeast extract was present to provide growth factors and may have also contributed as an electron donor. The ratio of total potential electron donor to electron acceptor was 468 mg COD/mg COD.

For treatments that were designed to enrich for sulfate-reducing conditions, the bicarbonate and phosphate-buffered media described by Freedman et al. (2002) was used. Sodium sulfide was used as a reductant. Yeast extract was provided as a source of growth factor but may have also been available as an electron donor, resulting in an electron donor to electron acceptor ratio of 12.2 mg COD/mg COD.

Two types of phosphate-buffered fermentative media were used, as previously described by Hata et al. (2003, 2004). Glucose was provided the primary electron donor to one type, along with an unusually high amount of yeast extract. The other medium received the same amount of glucose along with high amounts of L-asparagine and ferric citrate, but no yeast extract. Both had notably high levels of COD in comparison to the other media.

Table 2.1. Media used in microcosms and enrichments.[a]

	Type of Media						
Compound	**O_2/NO_3^-**	**Fe(III)-1**	**Fe(III)-2**	**Sulfate**	**Glucose**	**GLF**	**MSM**
K_2HPO_4	3880	-	-	870	-	3000	348
KH_2PO_4	-	-	-	680	280	800	-
$NaHCO_3$	-	2500	2500	1201	200	-	800
$(NH_4)_2HPO_4$	-	-	-	-	29	29	-
$NaH_2PO_4 \cdot H_2O$	1884	600	600	-	-	-	-
$(NH_4)_2SO_4$	2000	-	-	-	-	-	-
NH_4Cl	-	1500	1500	300	35	35	535
$MgCl_2 \cdot 6H_2O$	100	100	174	400	17	-	-
$MgSO_4 \cdot 7H_2O$	-	100	5	-	11	200	125
$CaCl_2 \cdot 2H_2O$	1	100	100	150	6	4	47
$FeSO_4 \cdot 7H_2O$	5	-	-	-	-	-	-
$FeCl_3 \cdot 6H_2O$	-	-	-	-	33	-	-
$ZnSO_4 \cdot 7H_2O$	2	-	-	-	-	-	0.42
$MnCl_2 \cdot 4H_2O$	1.23	5	5	10	-	-	2
$CuSO_4 \cdot 5H_2O$	0.2	-	-	-	-	-	-
$CoCl_2 \cdot 6H_2O$	0.4	-	-	19	0.8	-	3
EDTA Acid	10	-	-	-	-	-	-
$Na_2MoO_4.2H_2O$	0.2	1	1	3.6	-	-	-
KCl	-	100	100	300	30	-	-
NaCl	-	100	100	1000	-	-	-
$Na_2S \cdot 9H_2O$	-	-	-	120	-	-	240
$FeCl_2.H_2O$	-	-	-	-	-	-	162.6
$FeCl_2.4H_2O$	-	-	-	150.3	-	-	-
$ZnCl_2$	-	-	-	7	-	-	-
H_3BO_4	-	-	-	0.6	-	-	0.6
$NiCl_2.6H_2O$	-	-	-	2.4	-	-	1.5
$CuCl_2.2H_2O$	-	-	-	0.2	-	-	0.2
Na_2SeO_3	-	-	-	-	-	-	0.04
$Na_2SeO_3 \cdot 5H_2O$	-	-	-	0.3	-	-	-
$Al_2(SO_4)_3 \cdot 16H_2O$	-	-	-	-	-	-	0.2
Resazurin	1	1	1	1	1	1	1
Yeast Extract	-	50	50	50	2000	-	50
$NaCH_3COO$	-	2700	2700	-	-	-	-
D-Glucose	-	-	-	-	10000	10000	-
L-Asparagine	-	-	-	-	-	5000	-
Ferric Citrate	-	-	-	-	-	1000	-
$NaNO_3$	12.9	-	-	-	-	-	-
Fe(III)	-	44.7	44.7	-	-	-	-
Fe(III)-EDTA	-	44.7	44.7	-	-	-	-
Na_2SO_4	-	-	-	13.6	-	-	-
COD donor[b]	-	2991	2991	75	13700	16900	75
COD acceptor	6.07[c]	6.39	6.39	6.13	-	-	-
COD ratio[d]	-	493	493	12.3	-	-	-

[a] All values in mg/L unless otherwise specified. [b] The COD of yeast extract was assumed to be 1.5 mg COD/mg, presuming it was mainly protein. [c] The headspace of the O_2 treatment was purged with pure O_2. [d] COD ratio (mg COD/mg COD) is the quotient of the COD of donor divided by the COD of acceptor.

The medium described by Ettwig et al. (2010) (not shown in Table 2.1) was used to enrich samples from Site #7 microcosms, to test the hypothesis that anaerobic oxidation of VC may be coupled to nitrite or nitrate reduction.

2.3 EXPERIMENTAL DESIGN FOR MICROCOSMS AND ENRICHMENT CULTURES

Two main types of microcosms were prepared, depending on the size of the container: Balch tubes or serum bottles.

2.3.1 Balch Tubes

Sediment samples were obtained from a canal in Salem County, New Jersey entering the Delaware River from a site Zinder and colleagues have previously demonstrated reductive dehalogenation of chlorobenzenes (Fung et al., 2009; Nelson et al., 2011). Microcosms were inoculated using the sediment from a core sample 20-36 cm from water sediment interface obtained in May 2009 and stored at 4^{o} C in glass jars before use. The sediment below the oxidized top centimeter was black and fine-grained, and site analyses indicated the sample was freshwater and contained ca. 2 mM sulfate in the porewater. Electron acceptors were tested by amending sediment microcosms with 5 mM sodium sulfate, 5 mM sodium nitrate, or 5 mM amorphous iron oxide.

2.3.2 Serum Bottles

Microcosms were prepared with samples collected from 10 sites, as shown in Table 2.2. Each row in Table 2.2 is characterized by a site number, a set number (I, II, III, or IV), and a letter (A, B, C, or D). The set number refers to the time when the microcosms were prepared, i.e., all bottles within a given set number were prepared at the same time. The letters refer to variations in how the microcosms were prepared, either based on the type of groundwater used, the type of soil used (if any), and whether or not the headspace was purged prior to adding VC or ethene.

Site #1 was the location where anaerobic oxidation was previously reported in microcosms monitored by Cline (2003) and Pickens (2004). Several microcosms constructed by Cline with groundwater from well MW-6 and a nearby soil core exhibited apparent VC oxidation activity. Pickens observed apparent VC oxidation activity in microcosms constructed with groundwater from well MW-7 and a different soil core. However, as the results show, microcosms prepared for this study with groundwater from the same wells and a new set of core samples did not exhibit any evidence of VC oxidation. Although the cause was never determined, one possibility was that the zone of oxidative activity at the site had moved further downgradient, during the several year gap between when samples were collected for Cline and Pickens versus this study. It was, therefore, decided that new samples from the site should be collected further downgradient. Groundwater samples for Sets III and IV were obtained further downgradient of wells MW-6 and MW-7. Samples from five other sites were also collected, based on field data suggesting that anaerobic oxidation of VC may be occurring. By collecting samples from a variety of sites, the intent was to maximize the chances of finding VC oxidative behavior in at least one microcosm.

Table 2.2 Summary of microcosms evaluated.

Site	Set[1]	Prepared	Well	Soil Used	Micro. Type[2]	TEA Added[3]	Headspace Purging[4]	VOCs Other Than VC	Initial VC or ethene (μmol/btl)	Initial VC or ethene (mg/L)	Total No. of Microcosms	No. with [^{14}C]VC	No. with [^{14}C] ethene	No. serving as ACs[5]	No. serving as WCs[6]
#1	I-A	Apr-07	MW-6	no	Y	yes	no	TCE,cDCE DCM	12	5.0	60	0	0	3	0
#1	I-B	Apr-07	MW-7	no	Y	yes	no	-	12	5.0	60	0	0	3	0
#1	II-A	Jun-07	MW-6	yes[a]	X	no	no	cDCE, DCM	12	5.0	12	0	0	0	0
#1	II-B	Jun-07	MW-7	yes[a]	X	yes	no	-	12	5.0	60	0	0	0	0
#1	III-A	Oct-07	#8	no	Y	no	no	-	12	5.0	15	0	0	0	0
#1	III-B	Oct-07	#10	no	Y	no	no	-	12	5.0	15	0	0	0	0
#1	IV-A	Apr-08	#2114	yes[b]	X	no	no	TCE	1	0.44	18	6	0	3	0
#1	IV-B	Apr-08	#2114	yes[b]	X	no	yes	TCE	1	0.44	18	6	0	3	0
#1	IV-C	Apr-08	#2114	yes[c]	X	no	no	TCE	1	0.44	18	6	0	3	0
#1	IV-D	Apr-08	#2114	yes[c]	X	no	yes	TCE	1	0.44	18	6	0	3	0
#2	I-A	Sep-07	#92	no	Y	no	no	-	12	5.0	15	0	0	0	0
#2	I-B	Sep-07	#93	no	Y	no	no	-	12	5.0	15	0	0	0	0
#3	I	Nov-06	NS	yes	X	yes	no	-	12	5.0	38	5	0	0	3
#4	I-A	Mar-08	NS	ATP-6	X	no	no	-	1	0.44	18	6	0	3	0
#4	I-B	Mar-08	NS	ATP-6	X	no	yes	-	1	0.44	18	6	0	3	0
#4	I-C	Mar-08	NS	ATP-7	X	no	no	-	1	0.44	18	6	0	3	0
#4	I-D	Mar-08	NS	ATP-7	X	no	yes	-	1	0.44	18	6	0	3	0
#5	I-A	Mar-08	none	outcrop	Z	no	no	-	1	-	6	3	0	2	0
#5	I-B	Mar-08	none	outcrop	Z	no	yes	-	1	-	6	3	0	2	0
#5	I-C	Mar-08	none	KBA	Z	no	no	-	1	-	6	3	0	2	0
#5	I-D	Mar-08	none	KBA	Z	no	yes	-	1	-	6	3	0	2	0

Table 2.2, continued

Site	Set[1]	Prepared	Well	Soil Used	Micro. Type[2]	TEA Added[3]	Headspace Purging[4]	VOCs Other Than VC	Initial VC or ethene (μmol/btl)	Initial VC or ethene (mg/L)	Total No. of Microcosms	No. with [^{14}C]VC	No. with [^{14}C] ethene	No. serving as ACs[5]	No. serving as WCs[6]
#6	I-A	May-08	none	yes	Z	no	no	-	1	-	6	3	0	2	0
#6	I-B	May-08	none	yes	Z	no	yes	-	1	-	6	3	0	2	0
#7	I-A	Jun-09	E-14A	no	Y	yes	yes	cDCE, TCE	2	0.78	19	0	0	1	0
#7	I-B	Jun-09	TW-4A	no	Y	yes	yes	cDCE, TCE	2	0.78	19	0	0	1	0
#7	II-A	Aug-09	IP-05	no	Y	yes	yes	cDCE, TCE	2	0.78	19	0	0	1	0
#7	II-B	Aug-09	IP-07	no	Y	yes	yes	cDCE, TCE	2	0.78	19	0	0	1	0
#7	III-A	Nov-09	E-14A	no	Y	no	no	cDCE, TCE	2	0.78	4	0	0	0	0
#7	III-B	Nov-09	E-14A	no	Y	no	yes	cDCE, TCE	2	0.78	2	0	0	0	0
#8	I	Nov-09	none	yes	W	yes	yes	ethene only	2	0.073	24	0	0		
#8	II	Feb-10	none	yes	W	yes	yes	ethene only	2	0.073	38	0	13		
#9	I				X	yes	no	ethene only			2	0	0	0	0
#9	II		48; 50	yes	X	yes	No	ethene, ethane	41; 165	17.2; 6.0	96	0	0	12	0
#10	-	May-10	none	no	X	yes	no	ethene only			enrichments	0	0		
										TOTAL:	**712**				

[1]Sets refer to when a group of bottles was prepared; letters (A, B, C, D) refer to the type of microcosm preparation based on the groundwater used, soil used, and whether or not the headspace was purged.
[2]X = GW + soil; Y = GW only; Z = soil only; W = soil + medium.
[3]Terminal electron acceptors (TEA) tested = Fe(III), Fe(III)-EDTA, Mn(IV), sulfate, and AQDS.
[4]Purged with N_2 to remove H_2 present in the glovebox atmosphere.
[5]AC = autoclaved control.
[a] #SB12 47-55053107-0435; [b] MW-2118-72 #2219; [c] MW-2113-48 #2151.
NS = not specified.

Four sets of microcosms were prepared from Site #1. Set I was prepared with groundwater alone, from two different wells (MW-6 and MW-7). At the time these microcosms were prepared, soil samples were not yet available. Groundwater from MW-6 contained significant levels of TCE (10.8 mg/L), cDCE (11.3 mg/L), and DCM (54.2 mg/L), in addition to VC (5.98 mg/L) (averages calculated from Site I Set I-A unamended bottles at time zero). The groundwater from MW-7 did not contain significant amounts of any volatile organic compounds. Six treatments were prepared for each groundwater, with ten bottles per treatment: 1) no amendments, to simulate as-is conditions; 2) addition of amorphous Fe(III); 3) addition of EDTA-Fe(III) with the intent of increasing the bioavailability of Fe(III); 4) addition of amorphous Mn(IV); 5) addition of sulfate; and 6) addition of AQDS, to serve as a surrogate for humic acids.

The Set II microcosms for Site 1 utilized the same two groundwater samples (MW-6 and MW-7), although this time soil from a single core was also added. A complete array of microcosms was prepared with groundwater from MW-7 (i.e., ten replicates for the six treatments listed above). Due to limitations on the amount of soil available, plus the presence of co-contaminants in groundwater from MW-6, only one treatment was prepared, representing the as-is conditions (12 bottles). During the time groundwater from MW-6 was stored prior to preparing Set II, the background concentration of TCE decreased to nondetect, while cDCE and DCM decreased to 0.51 mg/L and 7.26 mg/L, respectively (averaged from all 12 Site #1 Set II-A bottles at time zero).

Set III microcosms for Site #1 were prepared with two groundwater samples, from monitoring wells #8 and #10, which are located further downgradient from MW-6 and MW-7. Soil was not available at the time of preparation. The only treatment tested for this set was without amendments (15 bottles per groundwater type).

Set IV microcosms for Site #1 were prepared with groundwater from a single well (#2114) and two types of soil samples. In addition, two methods of preparation were used. For subsets A and C, the headspace of the microcosms was not purged, while for B and D, the headspaces were purged to remove hydrogen from the bottles that was from the anaerobic chamber's atmosphere. For each subset (i.e., A, B, C and D), 15 live bottles were prepared without amendments, along with three autoclaved controls, for a total of 18 bottles per subset.

Microcosms from Site #2 consisted of first-flush groundwater from two wells (#92 and #93); soil was not available. A total of 15 live bottles were prepared with each groundwater sample, without amendments and without purging the headspace of the microcosms.

For Site #3, a total of 35 microcosms were prepared. All received soil and water from an unidentified well. Sufficient sample was available to permit preparation of ten unamended bottles (five received only unlabeled VC; five received unlabeled VC + [^{14}C]VC) plus five bottles for each alternate terminal electron acceptor (i.e., Fe(III), EDTA-Fe(III), Mn(IV), SO_4^{2-}, and AQDS).

For Site #4, two types of soil cores were received along with one type of groundwater. Two methods of preparation were used, resulting in four subsets of bottles (A, B, C and D). For subsets A and C, the headspace of the microcosms was not purged, while for B and D, the

headspaces were purged to remove hydrogen from the bottles that was from the anaerobic chamber's atmosphere. For each subset, 15 live bottles were prepared without amendments, along with three autoclaved controls, for a total of 18 bottles per subset.

Microcosms for Site #5 followed the same pattern as for Site #4, i.e., four subsets were prepared using two different types of soil and two types of preparation methods. For Site #5, however, no groundwater was used. Instead, all of the microcosms received 50 g of sediment + 6 mL of autoclaved DDI water. Also, enough soil was available to prepare only four live bottles and two autoclaved controls per subset, or 24 bottles in total.

For Site #6, only one type of soil was available; water was not available. Similar to Site #5, all of the microcosms received 50 g of sediment + 6 mL of autoclaved DDI water. Two subsets were prepared (A and B), with one set having the headspace purged and the other set not purged. Enough sediment was available to prepare only six bottles per subset, four of which were live and two of which were autoclaved.

For Site #7, only first flush groundwater was available, from four wells. Set I was prepared with groundwater from wells E-14A and TW-4A. The groundwater from well E-14A contained low levels of TCE (0.16 mg/L) and cDCE (0.75 mg/L) but no detectable level of VC (averages calculated based on unamended bottles at time zero). The groundwater from well TW-4A also contained low levels of TCE (0.11 mg/L) and cDCE (0.33 mg/L) and no VC (averages calculated based on unamended bottles at time zero). Due to the limited amount of groundwater available, duplicates rather than triplicates were prepared for each treatment.

Site #7, Set II microcosms were prepared with groundwater from wells IP-05 and IP-07, which are downgradient of E-14A and TW-4A. The groundwater from well IP-05 contained low levels of TCE (0.01 mg/L) and cDCE (0.18 mg/L), but no VC (averages based on unamended bottles at time zero). The groundwater from well IP-07 also contained low levels of TCE (0.07 mg/L), and cDCE (0.65 mg/L) and no VC (averages based on unamended bottles at time zero).

Based on apparent VC oxidative activity in several of the Site #7, Set I-A unamended microcosms, additional groundwater (also first flush) was requested and received from well E-14A. This new sample was used to prepare the Set III microcosms, which contained low levels of TCE (0.11 mg/L), cDCE (0.56 mg/L), and VC (0.002 mg/L) (averages based on the unamended bottles at time zero). For Set III, only unamended microcosms were prepared, since apparent activity in the Set I-A microcosms occurred only in the unamended treatment. Four of the bottles (i.e., Set III-A) were prepared by purging the headspace with N_2, as described in section 2.4.3. Two of the bottles (i.e., Set III-B) were purged with N_2 by inserting a cannula into the groundwater, with the intent of removing any dissolved oxygen that may have persisted following preparation of the bottles in the anaerobic chamber. Purging the groundwater stripped out the cDCE and TCE, so that only VC was present at time zero (Table 2.2).

Site #8 soil came from a flower bed outside of the L. G. Rich Environmental Research Laboratory in Anderson, SC. There is no record of prior contamination of the soil with chlorinated organic compounds. Sets I and II were prepared at different times, with different soil samples, and were therefore given different set numbers. Both sets were prepared with media rather than groundwater. For Set I, triplicates of the following treatments were prepared (in the

media listed in Table 2.1): 1) autoclaved controls, prepared with the O_2/NO_3^- medium; 2) addition of oxygen (to serve as a positive control) along with the O_2/NO_3^- medium; 3) addition of nitrate, using the O_2/NO_3^- medium; 4) addition of amorphous Fe(III), using the Fe(III)-1 medium; 5) addition of EDTA-Fe(III), using the Fe(III)-2 medium; 6) addition of sulfate, using the sulfate medium; 7) addition of glucose,, using the glucose medium; and 8) addition of glucose, L-asparagine, and ferric citrate (GLF), using the GLF medium. For Set II, the same eight treatments listed above for Set I were prepared, plus three additional treatments (also in triplicate): 1) addition of oxygen and VC instead of ethene, to test for aerobic VC bio-oxidation, prepared with the O_2/NO_3^- medium; 2) unamended, to simulate as-is conditions, prepared with the O_2/NO_3^- medium; and 3) addition of amorphous Fe(III) and VC instead of ethene, to test for VC bio-oxidation under iron-reducing conditions, prepared with the Fe(III)-1 medium.

Site #9 represents the Twin Lakes area at the Savannah River Site, where a groundwater plume contaminated with TCE discharges to a wetland and undergoes *in situ* reductive dechlorination to ethene and ethane. Set I consisted of two microcosms that were obtained from a previous project (Eaddy, 2008). In the course of the research for this report, the microcosms were provided with increasing amounts of ethene and sulfate, in an attempt to transition from reduction of ethene to ethane to anaerobic oxidation of ethene. Set II consisted of a new set of microcosms prepared with fresh samples of soil and groundwater, with equal volumes blended from samples taken in the vicinity of wells #48 and #50. A total of 32 treatments were prepared (Table 2.3). The treatments included autoclaved controls and live bottles with no amendments, and amendments with nitrate, Fe(III), Fe(III)-EDTA, AQDS, Fe(III) + AQDS, and sulfate. Within each of these eight sets, four treatments were prepared: only VC added, only ethene added, VC + ethene added, and ethene + ethane added. The intent of having a treatment with VC + ethene added was to determine if the presence of the reductive dechlorination product from VC would favor an oxidative pathway for VC biodegradation over a reductive pathway. Likewise, the intent of having a treatment with ethene + ethane was to determine if having the reduction product present would favor an oxidative pathway for anaerobic biodegradation of ethene. Prior microcosms prepared with samples from the Twin Lakes wetland have exhibited a propensity for VC reduction to ethene, and ethene reduction to ethane (data not shown).

Site #10 is an industrial facility in the southeastern US and contains a groundwater plume contaminated with PCE. A portion of the plume discharges to a wetland, where samples of soil and groundwater were taken. Microcosms for this site were initially developed by Hickey (2010), who observed complete dechlorination of PCE to ethene and ethane. For the purpose of this project, samples from the microcosms were transferred to MSM (Table 2.1), yielding six enrichment bottles. Three of these received only unlabeled ethene; the other three received ethene plus [^{14}C]ethene. The initial amount of ethene added was 12±0.10 µmol/bottle, along with lactate (0.11 mM).

2.4 PREPARATION OF MICROCOSMS

2.4.1 Balch Tubes

Microcosms were prepared inside an anaerobic glove box (Coy Laboratory Products (Grass Park, MI) in 27 mL crimp-top Balch tubes. To each tube 1 g of sediment (wet wt.) was added to 10 mL of anaerobic deionized water and sealed with a Teflon-coated grey butyl rubber stopper and

Table 2.3. Summary of treatments for the Site #9, SRS microcosms.[a]

Treatment[b]	VC	Ethene	Ethane	Nitrate	Sulfate	Fe(III)	Fe(III)-EDTA	AQDS
UN-VC	41.2	-	-	-	-	-	-	-
UN-ETE	-	165	-	-	-	-	-	-
UN-VC+ETE	41.2	165	-	-	-	-	-	-
UN-ETE+ETA	-	165	165	-	-	-	-	-
NO3-VC	41.2	-	-	82.4	-	-	-	-
NO3-ETE	-	165	-	396	-	-	-	-
NO3-VC+ETE	41.2	165	-	478	-	-	-	-
NO3-ETE+ETA	-	165	165	396	-	-	-	-
FeED-VC	41.2	-	-	-	-	--	412	-
FeED-ETE	-	165	-	-	-	-	1,978	-
FeED-VC+ETE	41.2	165	-	-	-	-	2,390	-
FeED-ETE+ETA	-	165	165	-	-	-	1,978	-
Fe-VC	41.2	-	-	-	-	412	-	-
Fe-ETE	-	165	-	-	-	1,978	-	-
Fe-VC+ETE	41.2	165	-	-	-	2,390	-	-
Fe-ETE+ETA	-	165	165	-	-	1,978	-	-
AQDS-VC	41.2	-	-	-	-	-	-	206
AQDS-ETE	-	165	-	-	-	-	-	989
AQDS-VC+ETE	41.2	165	-	-	-	-	-	1,195
AQDS-ETE+ETA	-	165	165	-	-	-	-	989
Fe+AQ-VC	41.2	-	-	-	-	412	-	206
Fe+AQ-ETE	-	165	-	-	-	1,978	-	989
Fe+AQ-VC+ETE	41.2	165	-	-	-	2,390	-	1,195
Fe+AQ-ETE+ETA	-	165	165	-	-	1,978	-	989
SO4-VC	41.2	-	-	-	51.5	-	-	-
SO4-ETE	-	165	-	-	247	-	-	-
SO4-VC+ETE	41.2	165	-	-	299	-	-	-
SO4-ETE+ETA	-	165	165	-	247	-	-	-
AC-VC	41.2	-	-	-	-	-	-	-
AC-ETE	-	165	-	-	-	-	-	-
AC-VC+ETE	41.2	165	-	-	-	-	-	-
AC-ETE+ETA	-	165	165	-	-	-	-	-

[a] Amounts shown are µmol/bottle.

[b] UN = unamended; ETE = ethene; ETA = ethane; NO3 = nitrate; FeED = Fe(III)-EDTA; Fe = Fe(III); AQ = AQDS; SO4 = sulfate; AC = autoclaved control.

aluminum crimp. For inoculated microcosms, 1 mL of slurry from an actively oxidizing microcosm was transferred using a syringe with an 18-gauge needle into a microcosm containing 1 g fresh sediment into 9 mL of deionized anaerobic water. The headspaces of all microcosms were flushed using 70% N_2 /30%CO_2 (Airgas, Certified Standard purity) to remove H_2 from the glove box atmosphere. Unless stated otherwise, all solutions were prepared anaerobically by flushing with high-purity N_2 gas. Microcosms were amended with 1.0 g/L sodium bicarbonate as a buffer, a vitamin solution (Maymó-Gatell et al., 1997), and 0.10-0.15 mL (0.4-0.6 mmol/L nominal concentration) gaseous ethene or, in initial studies VC, was added by syringe as an initial dose. Microcosms were incubated inverted and statically at 30°C in the dark.

2.4.2 Serum Bottles

Microcosms were prepared in 160 mL serum bottles inside an anaerobic chamber, containing an atmosphere of approximately 1.5% hydrogen and 98.5% nitrogen. Four types of microcosms were constructed, depending on the use of soil, groundwater, and medium:

1) with 20 g of soil plus 50 mL of groundwater (Site #1, Sets II and IV; Site #3; Site #4; Site #9, and Site #10);

2) with 100 mL of first-flush groundwater (Site #1, Sets I and III; Site #2; and Site #7);

3) with 50 g soil plus 6 mL of DDI water (Sites #5 and #6); and

4) with 20 g soil plus 50 mL of mineral medium (Site #8).

Serum bottles were sterilized by autoclaving prior to adding the soil and/or groundwater. Resazurin was added to the groundwater or mineral medium to serve as a redox indicator, at 1 mg/L. The exception to this was Site #5 Set I-D and Site #6 Set I-B, which did not receive resazurin, in accordance with a protocol developed by Bradley (personal communication, Paul Bradley to David Freedman). After adding the soil and/or groundwater, the serum bottles were capped with slotted grey butyl rubber septa or Teflon-faced red rubber septa and sealed with aluminum crimp caps. Grey butyl rubber septa are effective in retaining VC and nonchlorinated gases such as methane, ethene and ethane, for incubation periods in excess of one year (Verce et al., 2000). They tend to be less effective at retaining cDCE, TCE, and PCE (Verce et al., 2000).

Descriptions of the procedures used for adding VC and [^{14}C]VC, terminal electron acceptors, headspace purging to remove hydrogen, and preparation of autoclaved and water controls are provided in the following subsection. After preparing the microcosms, they were incubated in an inverted position (liquid and/or soil in contact with the septum) at room temperature (ca. 22-24°C), shielded from light (in boxes), and inside the anaerobic chamber (to reduce the possibility of oxygen diffusion through the septa).

2.4.3 Addition of VC, Ethene, Ethane, [^{14}C]VC, and [^{14}C]ethene

In most of the groundwater samples that were received, VC levels were either below detection or less than 0.1 mg/L. Consequently, it was necessary to add unlabeled VC to achieve the targeted initial concentrations of 0.44, 5.0, and 17.2 mg/L. The earliest sets of microcosms that were prepared received 5.0 mg/L; out of concern that this dose might be causing inhibition, later

microcosms received a lower dose. The lower initial amount of VC added is within the range used in previous studies by Bradley and Chapelle (1997). The highest dose added was for Site #9, based on prior results (Bratt et al., 2004) indicating a tolerance for VC at similar levels. The total amount of VC gas added took into account partitioning between the headspace and liquid, to achieve the targeted aqueous phase concentration.

Due to the large number of microcosms prepared, the high cost of the labeled material, and the additional challenges associated with adding the labeled material, only a subset received [^{14}C]VC. [^{14}C]VC was added to microcosms using a procedure similar to the one described by Cline (2003). The [^{14}C]VC was separated from the toluene in which it was diluted by gas chromatography to avoid any possible influence of the toluene on VC mineralization. [^{14}C]VC was added to the microcosms by injecting 3 µL of the stock solution (using a 10 µL liquid syringe) onto a Hewlett-Packard 5890 Series II GC. This quantity provided ca. 0.45 µCi per bottle (equivalent to ca. 10^6 dpm), a level that is sufficiently high to allow for detection of low levels of potential transformation products (see below). Separation of the VC and toluene was achieved on a stainless steel column (3.2 mm by 2.33 m) packed with 1% SP-1000 on 60/80 Carbopack B (Supelco, Inc.). The column and injector were both held at 200°C and the N_2 carrier was set at approximately 30 mL/min. Effluent from the column was routed through a 4-port valve out of the GC oven (rather than going to the detector) through stainless steel tubing (1.59 mm inside diameter), connected to a needle (22 gauge) at the end. During the time when VC eluted (0.6-1.2 min at 200°C), the needle was injected into a microcosm. The next addition of stock solution was not made for approximately one hour, to allow the comparatively large amount of toluene to elute from the column.

Addition of [^{14}C]VC to a microcosm increased the total amount of VC by approximately 0.2 µmol per bottle. The actual amount of ^{14}C label added to the microcosms was established by counting the activity in a headspace sample (0.5 mL) and liquid sample (100 µL) immediately after [^{14}C]VC addition (see "Analysis of ^{14}C-Labeled Compounds" below).

Prior to adding the [^{14}C]VC, 15 mL of headspace was withdrawn (using a 10 mL Pressure Lok syringe). Although the head pressure in the GC column (about 30 psi) was adequate to deliver the [^{14}C]VC into the headspace of the serum bottles, a 15 mL vacuum was pulled on each microcosm to avoid over-pressurizing the bottle, which could have increased the rate of diffusive loss of volatile compounds.

Microcosms developed with material from Sites #8, #9, and #10 were used to explore the potential for anaerobic oxidation of ethene. Since none of the soil or groundwater used contained a measurable level of ethene, it was added. Several of the treatments prepared with groundwater and soil from Site #9 also received ethane. The initial amounts added resulted in aqueous phase concentrations of 6.0 mg/L ethene and 2.8 mg/L ethane. [^{14}C]ethene was added to 13 of the Site #9, Set II microcosms, using [^{14}C]ethene that was generated by a reductive dechlorinating enrichment culture spiked with [^{14}C]VC.

2.4.4 Addition of Terminal Electron Acceptors to Microcosms

As shown in Table 2.2, many of the microcosms were amended with terminal electron acceptors. The amounts added were based on providing an excess of the electron equivalents (eeq) needed for stoichiometric oxidation of the initial amount of VC or ethene present. Ignoring cell synthesis, this amount is 10 µeeq per µmol of VC and 12 µeeq per µmol of ethene. Sulfate was added using a 0.048 mM stock of sodium sulfate. Fe(III) was provided using a 200 mM stock solution of amorphic Fe(III). Fe(III)-EDTA was added using a 0.384 mM stock solution. Mn(IV) was added via a 15 mM solution of MnO_2. AQDS was provided via a 0.192 mM of anthraquinone-2,6-disulfonic acid, disodium salt. Nitrate was a component in two types of media (O_2/NO_3^- shown in Table 2.1 and the medium described by Ettwig et al. (2010) (not shown in Table 2.1); additional amounts were added using stock solutions. Oxygen was added as a neat gas.

With many of the microcosms, the initial addition of VC consumed and more was subsequently added. More terminal electron acceptor was also added, at the same ratio as when the bottles were prepared. To maintain a constant amount of liquid in the bottles (i.e., 50 or 100 mL), the same volume of electron acceptor solution to be added was first withdrawn from the microcosm. Prior to removing liquid, the microcosms were shaken to dislodge soil from the septum. The bottles were then allowed to stand upright until the soil settled out. The appropriate volume of clarified liquid was then removed with a syringe. This sample was then available for measuring Fe(II), Mn(II), and sulfate.

2.4.5 Headspace Purging to Remove Hydrogen

For several sets of microcosms (Site #1, Set IV-B and IV-D; Site #4, Sets I-B and I-D; Site #5, Sets I-B and I-D; Site #6, Set I-B; Site #7, Sets I and II; and Sites #8, and #9), preparation of the microcosms included purging of the headspace after the bottles were removed from the anaerobic chamber. High purity nitrogen gas (passed through a buffered solution of 20% titanium(III) chloride (Science Lab) to scrub trace levels of oxygen) was used, approximately 850 mL/min for 10 min per bottle. Purging was repeated the following day. The intent of purging was to remove the hydrogen that was present in the atmosphere of the anaerobic chamber and hence the headspace of the microcosms. Removal of hydrogen was confirmed by GC analysis of headspace samples (see below) after the second purging event.

The microcosms were incubated for an additional three days before spiking with neat VC, ethene, and/or ethane, and, for a subset of the bottles, with [^{14}C]VC or [^{14}C]ethene.

2.4.6 Preparation of Autoclaved and Water Controls

Killed controls were prepared for several sets of microcosms (Table 2.2) by autoclaving bottles at 121˚C for 15 min on three consecutive days. VC or ethene was then added. A subset of the autoclaved controls also received [^{14}C]VC or [^{14}C]ethene, using the same procedure described above for the live bottles.

Water controls were prepared on the bench top (i.e., not in the anaerobic chamber) by adding 100 mL of DDI water to triplicate serum bottles, followed by 0.3 mL of neat VC gas. These controls

were incubated inside of the anaerobic chamber, at room temperature, in boxes to exclude light, and in an inverted position (liquid in contact with the septum).

2.5 ANAEROBIC ETHENE ENRICHMENT CULTURES BASED ON BALCH TUBE MICROCOSMS

A mineral salts medium (Adrian et al., 2000) was dispensed into 27 mL crimp-top tubes or 120 mL serum vials, receiving 10 mL and 50 mL, respectively, inside an anaerobic glovebox (Adrian et al., 2000). Tubes and vials were sealed with Teflon-coated butyl rubber stoppers and aluminum crimps and removed from the glovebox and autoclaved. The cultures received the same additions in the same proportions as the microcosms receiving ethene described above. Tubes and vials were incubated inverted and statically at 30°C in the dark.

2.6 ENRICHMENT, ISOLATION, AND QUANTIFICATION OF AN AEROBIC VC-UTILIZING MICROBE

Several years prior to the start of this SERDP-sponsored project, soil and groundwater were collected from a chloroethene-contaminated industrial site in southern California. High aqueous phase concentrations of trichloroethene and dichloromethane near the source area are indicative of residual dense, nonaqueous-phase liquid. Field data demonstrate that trichloroethene is undergoing reductive dechlorination, with accumulation of cDCE and lesser amounts of VC. Downgradient there is a mass loss of chlorinated ethenes that cannot be accounted for in the accumulation of ethene, suggesting the possibility that another biodegradation mechanism is playing a significant role, such as anaerobic oxidation of VC (L. Lehmicke, Personal Communication). Microcosms were prepared by adding 20 g of soil and 50 mL of groundwater collected from the source zone, to 160 mL serum bottles inside an anaerobic chamber. Microcosms were sealed with Teflon-coated red rubber septa. All microcosms received an initial dose of 0.05 mL (ca. 2 µmol) VC. These microcosms were incubated inside an anaerobic chamber and were removed for sampling.

Aliquots from one of these sediment microcosms were used to prepare a second-generation of microcosms (hereafter referred to as transfer #1), inside an anaerobic chamber. These transfer #1 vials were inoculated with 0.5 mL of material from an active sediment microcosm plus 99.5 mL of groundwater, in 160 mL serum vials sealed with gray butyl rubber stoppers. After 122 days of incubation, 10 mL samples these bottles were used to prepare a second transfer by combining with 90 mL of groundwater (hereafter referred to as transfer #2).

Material from the transfer #2 microcosms was used as inoculum for a set of enrichment cultures. In this case, 0.1 mL was inoculated into 10 mL of mineral salts medium (Maymó-Gatell et al., 1999) in 27-mL crimp-top culture tubes prepared inside an anaerobic chamber (Coy Industries (Grass Park, MI)). All culture tubes were sealed with Teflon-coated butyl rubber stoppers and removed from the anaerobic chamber. The headspaces of the culture tubes were flushed with 30% CO_2 and 70% N_2 to remove residual H_2 and adjust the pH of this bicarbonate-containing medium to near neutrality. The tubes were then amended with 0.1 mL each of sterile anaerobic solutions of 10% (w/v) sodium bicarbonate, 2% yeast extract, and vitamins (Maymó-Gatell et al., 1997). VC was added to the headspaces (0.1 mL) using a gas-tight syringe. Enrichment cultures received 5 mM of anaerobic electron acceptors: sulfate, amorphous Fe(III) oxide, nitrate, or nitrite. Addition of 3 mL of air to the tube headspaces created microaerobic conditions (ca. 3.7%

O_2). The tubes were incubated statically, inverted, and maintained in the dark at room temperature (ca. 20-22 ^oC). Enrichment cultures potentially showing VC utilization were transferred at 1% inoculum to the same mineral salts medium without the addition of yeast extract, for further enrichment and isolation.

For isolation of aerobic VC oxidizers under microaerobic conditions, 60 mm plates were prepared with the same medium formulation amended with 1.5% noble agar. Ten mL of the medium plus agar was dispensed aerobically into 27 mL crimp-top tubes, flushed with N_2, sealed with butyl rubber stoppers, followed by autoclaving. Sterile, anaerobic tubes were then moved into the anaerobic chamber that contained a 50^oC heat block to prevent premature solidification. Once tubes reached ca. 50^oC, vitamins were added and the molten agar was poured out into the 60 mm plates, which were allowed to solidify inside the anaerobic chamber. Plates were inoculated, by streaking, from VC oxidizing cultures once they had solidified. The plates were then placed inside a ca. 1 L glass anaerobic canning jar that was then sealed with a lid outfitted with a gas sampling port (Apolinario and Sowers, 1996) and were removed from the anaerobic chamber. The headspace was then flushed with a 30% CO_2/70% N_2 to remove residual H_2. Following this, 10 mL of VC and 110 mL of air were added through the sampling port to establish microaerobic conditions. Plates were incubated at room temperature in the dark for three weeks. Colonies on these plates were picked, resuspended in 1 mL sterile deionized water, and 0.1 mL was inoculated into 10 mL of the sterile mineral medium described above in 27 mL crimp top tubes, and 0.25 mL VC and 3 mL air were added.

The optical density (OD) of cultures was measured at 600 nm on a Spectronic 21 (Bauch & Lomb, Rochester, NY), which was compared to an uninoculated medium blank.

2.7 VOLATILE ORGANIC COMPOUNDS

2.7.1 Balch Tubes

VC and ethene in Balch tube headspaces (0.1 mL sample) was quantified using a Perkin-Elmer 8500 gas chromatograph with a flame ionization detector, as previously described (Maymó-Gatell et al., 1999). Calibrations based on aqueous standards with the same liquid and headspace volumes as the samples and over the concentration ranges were measured and are expressed as nominal concentrations (mmol/L of liquid volume) rather than aqueous concentrations to facilitate stoichiometric analyses. VC and ethene consumption in microcosm studies and early culture studies was compared with uninoculated samples to account for loss through stoppers and sampling.

2.7.2 Serum Bottles

Volatile organic compounds (TCE, cDCE, VC, DCM, ethene, ethane and methane) in serum bottles were monitored by headspace analysis using a Hewlett Packard Series II 5890 Gas Chromatograph (GC). The mass of each compound present in a bottle was determined by analysis of a 0.5 mL headspace sample, using a flame ionization detector (FID) in conjunction with a column (3.2 mm by 2.33 m) packed with 1% SP-1000 on 60/80 Carbopack-B (Supelco, Inc.). The carrier gas used was nitrogen at 25 mL/min. The same GC program described by Gossett (1987) was used: 60°C for 2 min, ramp to 150°C at 20°C/min, ramp at 10°C/min to

200°C, hold for 10 min. Depending on the number of chlorinated compounds present, the run time was shortened accordingly (e.g., if only VC was present, the program was stopped after 6.5 min).

The GC response to a headspace sample was calibrated to give the total mass of the compound (*M*) in that bottle. Assuming that the headspace and aqueous phases were in equilibrium, the total mass present was then converted to an aqueous phase concentration:

$$C_l = \frac{M}{V_l + H_C V_g} \tag{2.1}$$

where C_l = concentration in the aqueous phase (µM); M = total mass present (µmol/bottle); V_l = volume of the liquid in the bottle (50 mL in microcosms with soil present, 100 mL in microcosms without soil present); V_g = volume of the headspace in the bottle (99 mL in microcosms with soil present, 60 mL in microcosms without soil present); and H_c = Henry's constant (dimensionless) at 23°C, obtained from Gossett (1987) for TCE, cDCE and VC; Freedman and Herz (1996) for ethene and ethane; and Tchobanoglous et al. (2003) for methane.

Response factors were determined for microcosms with and without soil present. For those without soil present, 100 mL of DDI water was added to four serum bottles. Varying amounts of TCE, cDCE and DCM were added using a stock solution dissolved in methanol. VC, ethene, ethane, and methane were added as neat gases. The bottles were placed on a shaker table for at least one hour, to allow the liquid and gas phases to reach equilibrium. Headspace samples were injected onto the GC and the GC responses were plotted against the total amount present in the bottles.

For microcosms with soil present, response factors were determined by adding 50 mL of DDI water to four serum bottles. To account for the volume displaced by soil (10.5-12.5 mL), an equivalent volume of glass beads was added. As described above, varying amounts of the compounds were then added, the bottles were equilibrated on a shaker table, and headspace samples were analyzed.

2.8 Analysis of ^{14}C-Labeled Compounds

The total amount of ^{14}C activity in a serum bottle was determined by measuring the activity in a headspace sample (0.5 mL) and liquid sample (0.1 mL). Both samples were added to liquid scintillation vials containing 15 mL of liquid scintillation cocktail (ScintiSafe PlusTM 50%, Fisher Scientific). The caps of the scintillation vials were modified to permit direct injection of the samples into the vials, thereby avoiding volatilization losses. The liner on the caps were removed and a 3 mm hole drilled in the center. A Teflon-faced red rubber septum was then placed inside the cap and secured to the vial containing the cocktail. Headspace and liquid samples were injected into the scintillation vial through the hole in the cap and the septum.

^{14}C activity was measured using either a WALLAC 1415 Liquid Scintillation Counter or a Perkin Elmer Tri-Carb 2910TR Liquid Scintillation analyzer. The total amount of ^{14}C activity in a bottle was calculated based on:

$$Total\ dpm\ per\ microcosm = A \cdot V_l + B \cdot V_g \quad (2.2)$$

where A = dpm/mL in the liquid sample; V_l = volume of liquid (50 mL in microcosms with soil present, 100 mL in microcosms or enrichments without soil; 10 mL in Balch tubes); B = dpm/mL in the headspace sample; and Vg = volume of headspace (99 mL in microcosms with soil present, 60 mL in microcosms or enrichments without soil; 10 mL in Balch tubes).

In order to determine the distribution of products formed from transformation of [^{14}C]ethene or [^{14}C]VC, the products present in the gas phase and the liquid phase were evaluated as previously described (Freedman and Gossett, 1989). Briefly, ^{14}C-labeled compounds in the gas phase were quantified by analysis of headspace samples (0.5 mL, using a 1.0 mL Pressure Lok syringe), using a GC/combustion technique. The eluent from the Carbopack GC column was routed through a 4-port valve located in the GC oven to a quartz glass column located outside the oven to a combustion tube where the compounds were oxidized to $^{14}CO_2$ and then trapped in 3 mL of 0.5 M NaOH in a 10 mL glass test tube. Multiple test tubes were used, corresponding to the elution times of the target compounds. The NaOH in each tube was transferred to LSC for counting. The glass combustion tube (6.35 mm inside diameter x 381 mm) was filled with copper (II) oxide (Alfa Aesar, Inc.) held at 800°C by a tube furnace (21100 Tube Furnace, Thermolyne).

The total amount of ^{14}C activity in a serum bottle attributable to a specific volatile compound (M) was calculated as follows:

$$M = C_g(V_g + V_l \cdot H_C) \quad (2.3)$$

where C_g = ^{14}C activity for a specific compound measured from the Carbopack column for VC (see below for ethene) or Carbosieve column for CH_4 and ethane (see below for ethene; dpm/mL); V_g = volume of the headspace in the bottle (99 mL in microcosms with soil present, 60 mL in microcosms or enrichments without soil present; 10 mL in the Balch tubes); V_l = volume of the liquid in the bottle (50 mL in microcosms with soil present, 100 mL in microcosms or enrichments without soil present; 10 mL in the Balch tubes); and H_C = Henry's Law constant (dimensionless) at 23°, using values from the following sources: Gossett (1987) for VC, Freedman and Herz (1996) for ethene and ethane, and Tchobanoglous et al. (2003) for methane.

For ethene, a modified approach was needed. The dpm for ethene was obtained by using the total dpm in the 0- to 1.5-min fraction off the Carbopack-B column (which included methane, ethene, ethane and CO_2) and subtracting the dpm for CH_4, CO_2, and ethane from dpm obtained for these compounds during analysis with the Carbosieve S-II column. Although it would have been more direct to simply use the dpm for ethene that eluted from the Carbosieve S-II, this column retained a portion of the ethene. Even after priming with neat ethene, ethene already on the column exchanged to some extent with [^{14}C]ethene in the sample, resulting in retention of [^{14}C]ethene on the column. The subtraction procedure described above consistently yielded a better mass balance for ^{14}C activity.

The efficiency of the combustion technique was determined by dividing the total ^{14}C activity in all fractions from the Carbopack column collected through the combustion tube by the total ^{14}C activity based on a direct count of a headspace sample (0.5 mL or 1.0 mL). Based on four

measurements, the average efficiency was 119±7.5%. The fact that the recovery was over 100% suggests that [^{14}C]ethene in the headspace sample added directly to liquid scintillation cocktail didn't completely dissolve, resulting a lower than expected denominator for the recovery calculation.

In order to measure the ^{14}C activity present in the aqueous phase, a modification of the procedure described by Freedman and Gossett (1989) was used. A 10 mL sample of well-mixed liquid was transferred from a bottle using a glass syringe to a 40 mL test tube (referred to as the stripping chamber). The stripping chamber was weighed (to the nearest 0.1 mg) and then connected to a second test tube (referred to as the trapping chamber, the mass of which was also determined) that contained 10 mL of 0.5 M NaOH. High purity nitrogen gas was sparged (50-60 mL/min) into the stripping chamber through a porous glass diffuser and then through latex tubing into the trapping chamber, where the gas was sparged through a glass filter stick (135 mm POR C, Ace Glass, Inc.). The contents of the stripping chamber were acidified by injecting 0.25 mL of 6 M HCl through a small hole near the base of the test tube, which was covered with a gray butyl rubber septum held in place by a hose clamp. Sparging continued for 30 min. The low pH in the stripping chamber converted all carbonates to CO_2, which was then stripped by N_2 and carried over to the trapping chamber, where the high pH converted CO_2 to soluble carbonates.

After sparging, the mass remaining in each chamber was used to calculate the loss due to evaporation, which was minimal (1.6±0.2%). Next, the ^{14}C activity in the stripping and trapping chambers was measured by transferring a 2.0 mL sample of each chamber to 10 mL of liquid scintillation cocktail. ^{14}C activity in the stripping chamber is referred to as non-strippable residue (NSR); ^{14}C activity in the trapping chamber is presumptively considered to be $^{14}CO_2$. In samples that contained more than 2% $^{14}CO_2$, the following test was performed to verify that the activity was indeed $^{14}CO_2$ and not something else that may have been retained in the trapping chamber. The remaining 8.0 mL in the trapping chamber was transferred to a 15 mL conical bottom, screw-top plastic centrifuge tube; 1.4 g of $Ba(OH)_2$ was added and the contents were vortexed for 60 s, with the intent of precipitating all of the carbonates as barium carbonate. The tubes were then centrifuged (Sorvall Superspeed RC2B, 10,000 rpm, 30 min). A 2.0 mL sample was carefully removed (to avoid resuspending any of the precipitate) and added to 10 mL of liquid scintillation cocktail. When the concentration of ^{14}C activity in the centrate was less than 2% of the contents in the trapping chamber, it was assumed that the trapping chamber contained only $^{14}CO_2$.

The percent distribution of ^{14}C in a bottle for each compound or NSR (C) was:

$$\%C = x/T_o \times 100 \tag{2.4}$$

where x = ^{14}C per bottle for compound x; and T_o = the total ^{14}C added per bottle at time zero. The amount of ^{14}C lost to diffusion and adsorption during incubation was:

$$\%Loss = (T_o - T_f)/T_o \times 100 \tag{2.5}$$

where T_f is the total ^{14}C remaining in a bottle at the final sampling time. The amount of ^{14}C in the "unaccounted for" (UAF) category was:

$$\%UAF = (T_f - \Sigma C)/T_o \times 100 \quad (2.6)$$

where ΣC = sum of the ^{14}C associated with all of the identifiable compounds or categories (CO_2, NSR, VC, ethene, ethane, or methane).

2.9 HYDROGEN AND OXYGEN

Hydrogen and oxygen were monitored by headspace analysis using a Hewlett Packard Series II 5890 GC equipped with a thermal conductivity detector (TCD) in conjunction with a Carbosieve SII (Supelco, Inc.) stainless steel column (3.175 mm x 3.048 m). The carrier and reference gas used was nitrogen, at flow rates of 24 and 50 mL/min, respectively. The column temperature was held constant at 105°C and the injector and detector temperatures were set to 200°C. The TCD sensitivity was set to high. Before taking samples (0.5 mL/bottle), the syringe and needle were flushed three times with high purity nitrogen gas, which was scrubbed of trace levels of oxygen by bubbling it through a buffered solution of 20% titanium(III) chloride. Attention was given to minimizing the time the syringe needle was exposed to room air before and after taking the sample and injecting it onto the GC, to minimize diffusion of oxygen into the void volume of the needle.

Nitrogen is not the preferred carrier gas for quantifying oxygen on a TCD, since they have similar thermal conductivities. The use of nitrogen was mainly due to the high percentage of nitrogen in the headspace of the microcosms, since they were purged with nitrogen gas as part of the preparation protocol (section 2.4.3). If a carrier gas other than nitrogen was used, it would have resulted in a large peak relative to oxygen, possibly "overwhelming" the TCD.

The GC response to a headspace sample was calibrated to give the total mass of the compound (*M*) in that bottle, as described for the volatile organic compounds. Response factors were determined for two conditions: 1) with 100 mL of DDI water per bottle and (for Site #7 microcosms); and 2) with 11 mL glass beads and 50 mL DDI water (for Site #8 microcosms). Serum bottles were purged with high purity nitrogen, placed on a shaker table for 1 hour to allow the liquid and gas phases to reach equilibrium, and analyzed to ensure that hydrogen and oxygen were below their detection limits. Varying amounts of oxygen and hydrogen were then added using a 1.0 mL and 10.0 mL Pressure Lok syringe, and again placed on a shaker table (minimum of 1 h) to allow the headspace and aqueous phases to reach equilibrium. GC responses were plotted against the total amount present in the bottles. The detection limit for hydrogen was 0.4 µmol/bottle. The quantification limit for oxygen was 2.0 µmol/bottle.

2.10 NITRATE AND SULFATE

Nitrate and sulfate were quantified using a Dionex DX-100 Ion Chromatograph (IC) (Sunnyvale, CA). A degassed sodium carbonate eluent (9mM) was used with a Dionex guard column (AG9-HC, 4 mm x 50 mm), followed by an IonPac® AS9-HC anion-exchange column (4 mm x 250 mm), at a flow rate of 1.0 mL/min. Samples (25 µL) of settled liquid from the microcosms were filtered through a 0.20 µm filter (PTFE, NALGENE®) and injected onto the IonPac column, using a sample volume of 25 µL. Response factors were developed using standard solutions of sodium sulfate and sodium nitrate.

When resolution problems developed with the AS9-HC column (May 2008), it was replaced with a Dionex guard column (IonPac® AG15, 4 x 50 mm) and an IonPac® AS15 column (4 mm x 250 mm). Sodium hydroxide (38 mM) was used as the eluent, at a flow rate of 1.2 mL/min.

2.11 IRON(II), MANGANESE(II), AND AQDS

The extent of Fe(II) accumulation in bottles amended with amorphic Fe(III) and EDTA-Fe(III) was determined using the ferrozine method, as previously described (Stookey, 1970). The procedure is based on reaction of Fe(II) with ferrozine, forming a complex that is quantified based on absorbance. Samples of clarified liquid from the microcosms were prepared by filtration (0.2 µm PTFE filter, NALGENE®). The filtrate (0.5 mL) was collected in a test tube. Acetate buffer (0.4 mL) and ferrozine reagent (0.1 mL) were added next. The contents were mixed well and then allowed to sit for 2-5 min for full color development before reading the absorbance on a Cary 50 Bio UV-Visible Spectrophotometer at a wavelength of 560 nm. Standard curves were used to calculate Fe(II) concentration up to 20 mg/L.

The extent of Mn(II) accumulation in bottles amended with amorphic Mn(IV) was determined using a procedure described by Serrat (1998), modified to permit use of smaller sample volumes. The procedure is based on oxidation of Mn(II) to Mn(IV), which is then reacted with 3,3',5,5'-tetramethylbenzidine, forming a complex that is quantified based on absorbance. Samples of clarified liquid from the microcosms were prepared by filtration (0.2 µm PTFE filter, NALGENE®). Preliminary tests demonstrated that Mn(IV) does not pass through the filter. The filtrate (1.0 mL) was collected in a test tube, followed by addition of 1.5 M NaOH (40 µL). The contents were allowed to stand for 15 min. An acidified solution of 3,3',5,5'-tetramethylbenzidine was then added, followed by 40 µL of 7.5 M phosphoric acid. The absorbance was measured on a Cary 50 Bio UV-Visible Spectrophotometer at a wavelength of 450 nm. Standard curves were used to calculate Mn(II) concentration up to 8 mg/L.

Reduced AQDS (AH_2QDS) was quantified spectrophometrically at 450 nm and compared against standards of chemically reduced AH_2QDS, as described by Lovley et al. (1996).

2.12 ORGANIC ACIDS

For the Balch tube experiments, organic acids such as lactate, succinate, and volatile fatty acids were determined by high performance liquid chromatography (HPLC) as previously described (Nelson et al., 2011) and had a detection limit of 10-20 µM. For the serum experiments, acetate was quantified using a Waters 600E High Performance Liquid Chromatograph (HPLC) system composed of an autosampler (Waters 717 plus), pumping system (Waters 600), and a UV/Vis detector (Model 490E) set at 210 nm. A degassed sulfuric acid eluent (0.01 N) was used with an Aminex® HPX-87H ion exclusion column (300 mm x 7.8 mm; BioRad) at a flow rate of 0.6 mL/min. Samples (50 µL) of settled liquid from the microcosms were filtered (0.45 µm PTFE, NALGENE®) and injected onto the Aminex column, using a sample volume of 50 µL. Response factors were developed using standard solutions of sodium acetate.

2.13 DOC ANALYSIS

Dissolved organic carbon (DOC) was measured with a Shimadzu Total Organic Carbon Analyzer (TOC-VCSH/CSN). Samples (1 mL) of clarified liquid were removed from microcosms, filtered (2 µm PTFE filter, NALGENE®), diluted with DDI water as needed, and injected (1 mL sample volume) onto the instrument. Standard solutions (0-15 mg/L DOC) were prepared by diluting a 1000 mg C/L standard stock solution made by diluting 2.125 g of reagent grade potassium hydrogen phthalate previously dried at 105-120°C for one hour and cooled in a desiccator in a 1 L volumetric flask with DDI water

2.14 CHEMICAL OXYGEN DEMAND

Bioscience, Inc. low-range twist cap vials (range 5-150 mg COD/L) were used to measure the chemical oxygen demand (COD). Samples (0.25-2.5 mL) of clarified liquid were removed from microcosms, filtered (0.2 µm PTFE filter; NALGENE®), diluted with DDI water as needed for a total sample volume of 2.5 mL, and added to the COD vials. Standards were prepared using a 10 g/L COD stock solution of potassium acid phthalate (made by adding 8.5034 g to a 1000 mL volumetric flask and diluting to volume with DDI water). The stock solution was diluted to provide five or six samples in the 5-150 mg/L range. After capping the vial, the contents were gently mixed, placed on a pre-heated heating block (150°C) for two hours, then removed from the heating block and allowed to cool to room temperature. The absorbance was then measured at 440 nm on a Milton Roy Spectronic 20D spectrophotometer and the COD was calculated using a standard curve.

In microcosms with sulfate added, there was a concern that sulfide generated from sulfate reduction would contribute to the measured COD. To remove sulfide, it was first precipitated with zinc prior to filtration (0.2 µm PTFE filter; NALGENE®). Assuming a sulfide concentration of 151.6 mg/L and stoichiometric reaction of the sulfide with zinc, the amount of zinc added to a 1.0 mL sample was 4.73 µmol. This was accomplished by adding 50 µL of a stock solution of zinc chloride (12.89 g/L $ZnCl_2$).

2.15 SULFIDE

Sulfide concentrations were determined using a colorimetric assay in which sulfide is reacted with N,N dimethylphenylenediamine which is then further oxidized by Fe(III) to methylene blue (Cline, 1969). Absorbance was measured at 670 nm on a DU730 LifeScience UV/Vis Spectrophotometer (Beckman Coulter, Indianapolis, IN), which was calibrated to an uninoculated medium blank. Both headspace and liquid samples were taken from the enrichment cultures to determine sulfide concentrations, and compared to standards of known concentration.

2.16 DNA EXTRACTION, 16S RRNA GENE CLONE LIBRARIES AND PHYLOGENETIC ANALYSES

2.16.1 Anaerobic Ethene Enrichment Culture

A cell pellet from 0.5 mL enrichment culture was used to extract DNA using the UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) following manufacturer's protocol.

A 16S rRNA gene clone library was constructed with amplifications from DNA extracted from the sixth generation ethene enrichment cultures using the universal primers 27F and 1392R. The PCR reaction mixture contained 1.25U 5PRIME Taq polymerase, 1X 5PRIME Taq buffer, 0.3µM, 200µM dNTPs, and 2.5 µL of extracted DNA in a total volume of 35µl. All PCR reactions were initiated a two minute 95 ℃ step followed by 30 cycles of 95°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min followed by a 10 min hold at 72°C and a hold at 4°C until analysis. PCR amplicons were analyzed on 1.25% TBE gels stained with ethidium bromide. PCR products were cloned with the Topo TA Cloning Kit into pCR2.1 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Inserts were PCR amplified using primers M13F and M13R. Inserts of the expected size were sequenced at the Life Sciences Core Laboratory Center at Cornell University.

For phylogenetic analyses, 16S rRNA gene sequences were aligned using the online Silva aligner (www.arb-silva.de) (Pruesse et al., 2012). Sequences were trimmed to the same length resulting in 1282 positions in the final dataset. The resulting alignment was then used to create a phylogenetic tree using a maximum likelihood method based on the Kimura 2-Parameter model within the MEGA 5.1 software package (Tamura et al., 2011).

2.16.2 Aerobic VC Enrichment Cultures and Isolates

DNA from several of the groundwater microcosms and liquid cultures was extracted with a Power Soil DNA extraction kit (MoBio) using the manufacturer's protocol. DNA from the aerobic VC isolate was purified using an UltraClean Microbial DNA extraction kit (MoBio) using the manufacturer's protocol. 16S rRNA genes were amplified using universal bacterial primers 27F and 1492R (Felsenstein, 2005). The PCR reaction contained 1.25 U 5PRIME Taq polymerase, 1X 5PRIME Taq buffer, 0.3 µM primers, 200 µM dNTPs, and 2.5 µL of extracted DNA in a total volume of 35 µL. The PCR program consisted of 2 min at 95°C, 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, then a final elongation step at 72°C for 10 min. PCR products were examined by electrophoresis on 1% agarose gels in TBE buffer.

A clone library of the bacterial 16S rRNA genes in the groundwater microcosms was constructed as previously described (Cadillo-Quiroz et al., 2006). PCR products of the16S rRNA gene from four different VC oxidizing microcosms were pooled for cloning. Cloned DNA was amplified with M13 primers by the same PCR method as above and the PCR products were digested with HaeIII and HhaI (NEB, Ipswitch, MA) for restriction fragment length pattern analysis. Digestion mix consisted of 1U of each enzyme, 1X NEBuffer 2 and 10 µL of PCR product. Reaction proceeded overnight at 37°C. RFLP patterns were examined on 2% agarose gels in TBE buffer. The samples corresponding to unique patterns by RFLP and VC oxidizing isolate were prepared for sequencing by reaction with ExoSap Enzyme (Affymatrix, Santa Clara, CA) and sequenced at the Cornell University Life Sciences Core Laboratories Center using Sanger sequencing.

Sequences were compared to those in GenBank by BLAST analysis, and related sequences were downloaded for further analysis. The sequences were aligned using the online Silva aligner (www.arb-silva.de) (Pruesse et al., 2012). The resulting alignment was then used to create a phylogenetic tree using a maximum likelihood method based on the Kimura 2-Parameter model within the MEGA 5.2 software package (Tamura et al., 2011).

2.17 QUANTITATIVE PCR

Quantitative PCR (qPCR) amplifications were performed in triplicate using a MyiQ Single Color Real Time Detection System (Bio-Rad). Reaction mixtures (final volume 25 μL) contained 12.5 μL iQ SYBR Green Super Mix (BioRad, Hercules, CA), forward and reverse universal eubacterial primers (Nadkarni et al., 2002) (200 nM each) and 1 μL of template DNA. Cycling condition were: 10 min at 95°C, 35 cycles of 15 s at 95°C and 1 min at 62°C, followed by melting curve analysis from 60 to 95°C to screen for primer dimers. Total bacterial 16S rRNA gene copy numbers were determined by analyzing a dilution series of a known quantity of a plasmid containing the appropriate part of the 16S rRNA gene from *Bacillus subtilis* strain 168 (Nelson et al., 2011). DNA concentrations were estimated spectrophotometrically using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE).

3.0 RESULTS: OVERVIEW

To address the objectives of this project, samples from numerous sites (and many locations for each site) were evaluated for anaerobic oxidation of VC and/or ethene. With samples of sediment from one site in Salem County, New Jersey, definitive evidence was obtained for anaerobic oxidation of ethene under sulfate reducing conditions. An enrichment culture was developed in a defined anaerobic mineral medium and was transferred numerous times with ethene as the sole source of organic carbon and energy. Analysis of the enrichment suggests that the microbe responsible for anaerobic growth on ethene is *Desulfovirga adipica*. This is an important development since identification of a microbe capable of anaerobic oxidation of ethene affords an opportunity to develop tools to evaluate the significance of this activity *in situ*. The results for this part of the project are presented in section 4.

For all of the other sites evaluated, the results were either negative (i.e., there was no evidence to support anaerobic oxidation, or the predominant biotic activity was reductive) or equivocal (i.e., consumption of VC or ethene occurred without accumulation of reductive daughter products, but the possibility of oxygen leakage could not be ruled out as an explanation). The results for this part of the project, covering more than 700 microcosms plus attempts to enrich activity in several presumptively positive microcosms, are presented in section 5.

The final set of results are presented in section 6. The original set of microcosms developed by Cline (2003) (section 1.1.4) that exhibited presumptive evidence of anaerobic VC oxidation were evaluated further. Although the microcosms sat idle for several years, VC biodegradation activity was restored, although only in microaerobic treatments. A clone library of the microaerobic microcosms was dominated by mycobacteria. These results support the proposition that VC biodegradation *in situ* may occur under microaerophilic conditions and thereby contribute to the overall lack of mass balances for chlorinated ethenes.

4.0 RESULTS AND DISCUSSION: ANAEROBIC OXIDATION OF ETHENE

4.1 ANAEROBIC OXIDATION OF ETHENE IN SEDIMENT MICROCOSMS

Microcosms constructed from a sample from a freshwater site we have previously studied (Fung et al., 2009; Nelson et al., 2011) did consume ethene, but not VC, when amended with sulfate as the electron acceptor. Figure 4.1 shows a typical microcosm amended with sulfate. Ethene was depleted within 77 days, and subsequent doses of ethene were consumed at a higher rate, indicative of a biologically mediated reaction. Consumption of ethene was somewhat slower in the microcosms not amended with sulfate in comparison to the sulfate amended microcosms with the first dose of ethene consumed after 88 days and second dose consumed after 14 more days. Sulfate could have been the terminal electron acceptor in all these cases since the unamended microcosms likely contained 1.8 mmol/L sulfate (based on previous studies of this site), enough to consume the doses added here. Microcosms amended with Fe(III) oxide showed oxidation rates similar to those in the unamended microcosms and no ethene consumption was found in nitrate-amended sediments (Figure A-1). No methane or ethane was detected in the microcosms.

The addition of sediment slurry (ca.1% v/v) that had consumed ca. 2 mmol/L of ethene to fresh sediment microcosms showed accelerated ethene oxidation activity in comparison to the "naïve" sediment microcosms (Figure A-2).

4.2 DEVELOPMENT OF ETHENE OXIDIZING ENRICHMENT CULTURES

Sediment slurries from microcosms actively consuming ethene were transferred into a mineral salts medium with amorphous iron sulfide as the reducing agent and a vitamin solution. Fermentable substrates such as Casamino Acids or yeast extract were not added to the enrichment cultures so that ethene was the sole electron donor and no other carbon sources besides CO_2 and trace amounts of vitamins were present.

First generation enrichment cultures were inoculated with 1% or 10% (v/v) of sediment slurry from a sulfate amended microcosm that had consumed 2 mmol/L of ethene. This enrichment culture inoculated at 10% consumed its first dose of ethene by 24 days, whereas the culture with1% inoculum required 63 days to consume its first dose (Figure 4.2A). Upon subsequent additions of ethene, consumption rates increased indicating growth of ethene oxidizing organisms. After day 91, the enrichment culture inoculated at 10% seemed to stall indicating that all sulfate had been consumed, since Equation 1.5 predicts that 5 mM sulfate can oxidize 3.3 mmol/L ethene, and ca. 3.4 mmol/L ethene had been consumed by day 91.

Second and third generation transfers were inoculated at 10% and 1%. In a third generation culture inoculated at 10%, the first dose of ethene was consumed in 12 days (Figure 4.2B). Abiotic controls and inoculated cultures lacking sulfate failed to consume ethene. Additions of increasing amounts of ethene did not reduce consumption rates.

qPCR using "universal" bacterial 16S rRNA gene primers was used to monitor growth in the fourth generation enrichment cultures. These enrichments received a 2% (v/v) inoculum from a third generation enrichment. Ethene consumption started after a lag of approximately 20 days (Figure 4.3A). Changes in bacterial 16S rRNA gene copies were tracked at inoculation and after

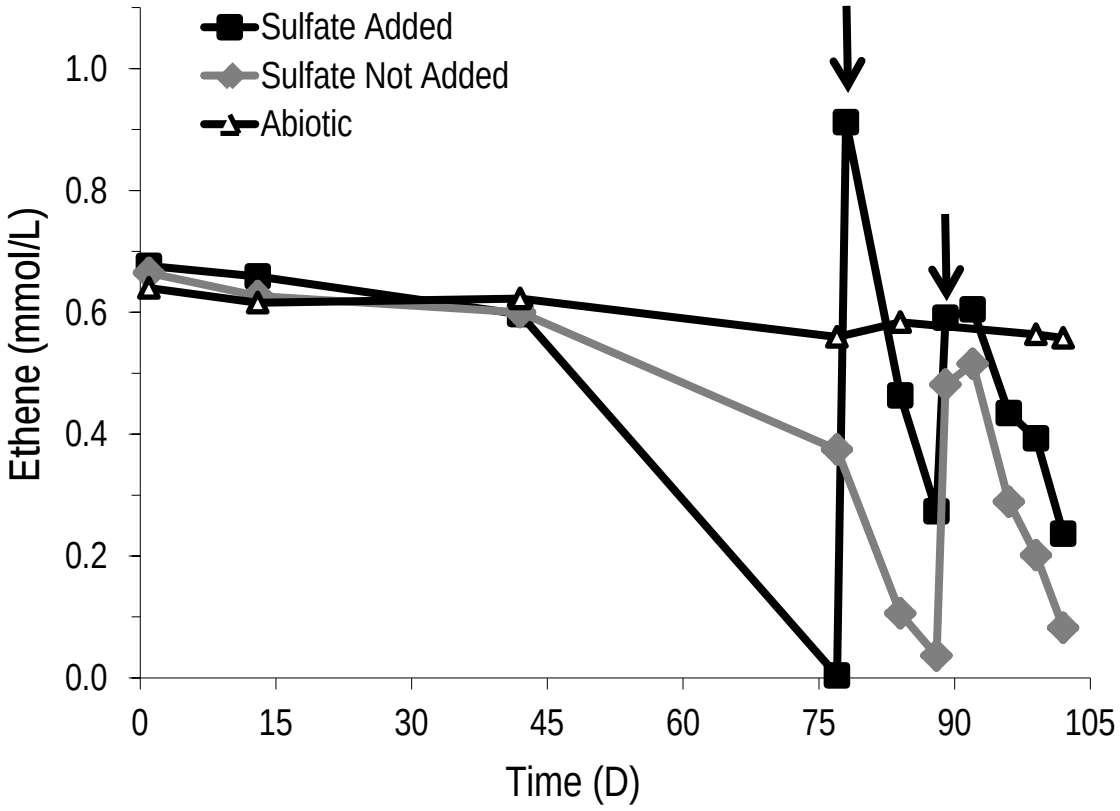


Figure 4.1. Anaerobic consumption of ethene in microcosms amended with 5 mM sulfate or unamended in comparison with an uninoculated water blank (abiotic). Arrows represent an addition of ethene. Representative tubes of triplicates for each treatment are shown (Figure A-1 presents replicates).

45 and 64 days of incubation (Figure 4.3B). In the culture not amended with sulfate, 16S rRNA gene copies per mL increased from 3.0×10^5 to 2.9×10^7 by day 64. The culture lacking ethene showed a similar increase in 16 rRNA copy number per mL from 3.6×10^5 to 1.8×10^7. However, this is much less than the culture amended with both ethene and sulfate in which 16S rRNA gene copies increased from 2.5×10^5 to 1.7×10^8. During this growth experiment, sulfide concentrations were also monitored (Figure 4.3C). In cultures lacking ethene or sulfate, there was little change in sulfide from background levels. However, in cultures amended with ethene and sulfate, sulfide production was evident, with 2.4 mmol/L sulfide produced compared to the sample lacking ethene and consumption of 1.3 mmol/L ethene (Figure 4.3A, 4.3C). The final molar ratio of 1.8 sulfide:ethene was somewhat higher than the 1.5 ratio predicted by Equation 1.5.

The fourth generation cultures amended with VC did not consume it over the course of 180 days (data not presented). Microcosms amended with Fe(III) or NO_3^- did not consume ethene, although cultures transferred successfully with thiosulfate as the electron acceptor (data not presented).

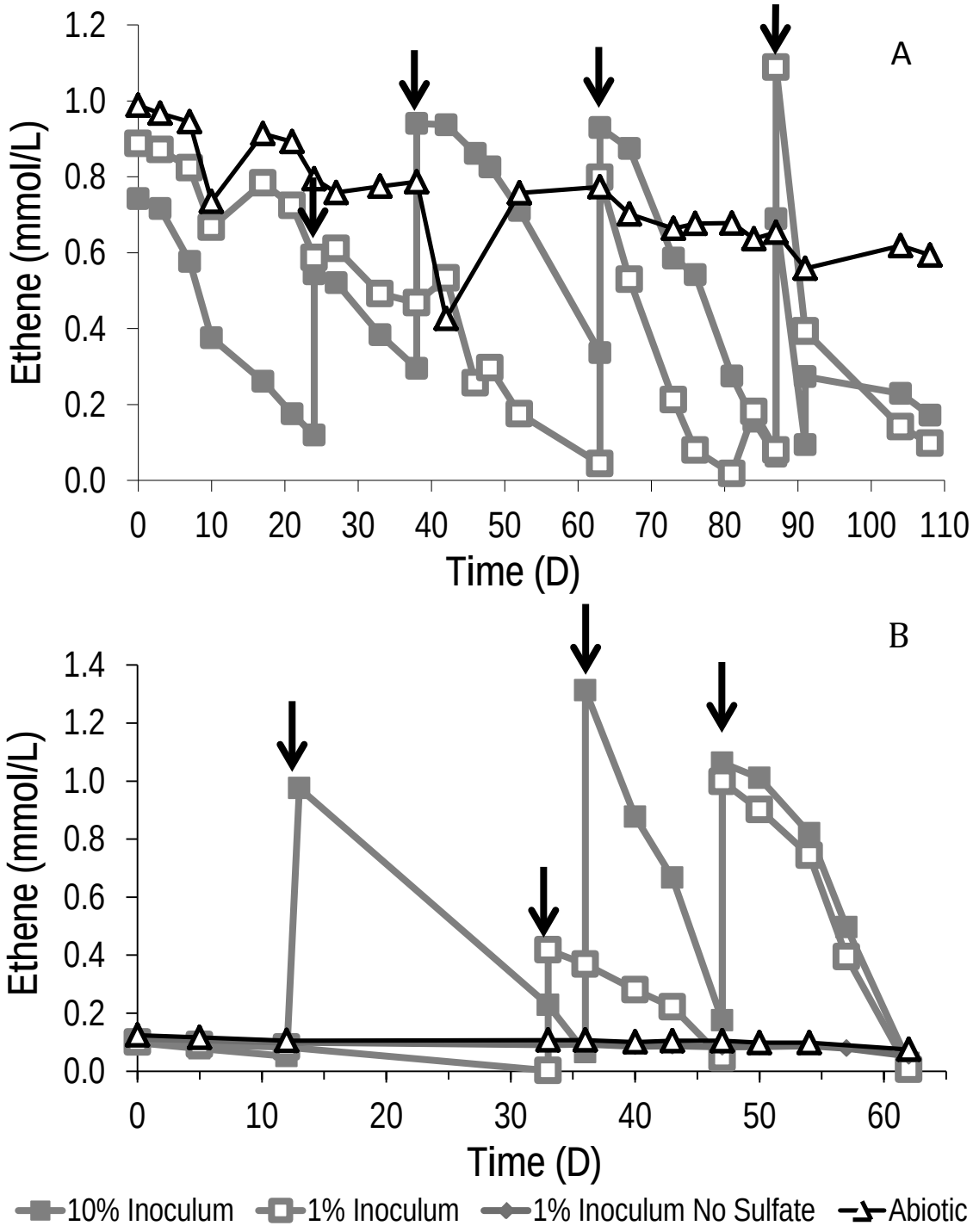


Figure 4.2. (A) First generation enrichment culture inoculated with either a 10% or 1% (vol/vol) sediment slurry from ethene-oxidizing microcosms amended with sulfate (Figure A-3 presents replicates). (B) Third generation enrichment cultures. Arrows represent an addition of ethene. One representative tube out of a triplicate set is shown (Figure A-4 presents replicates).

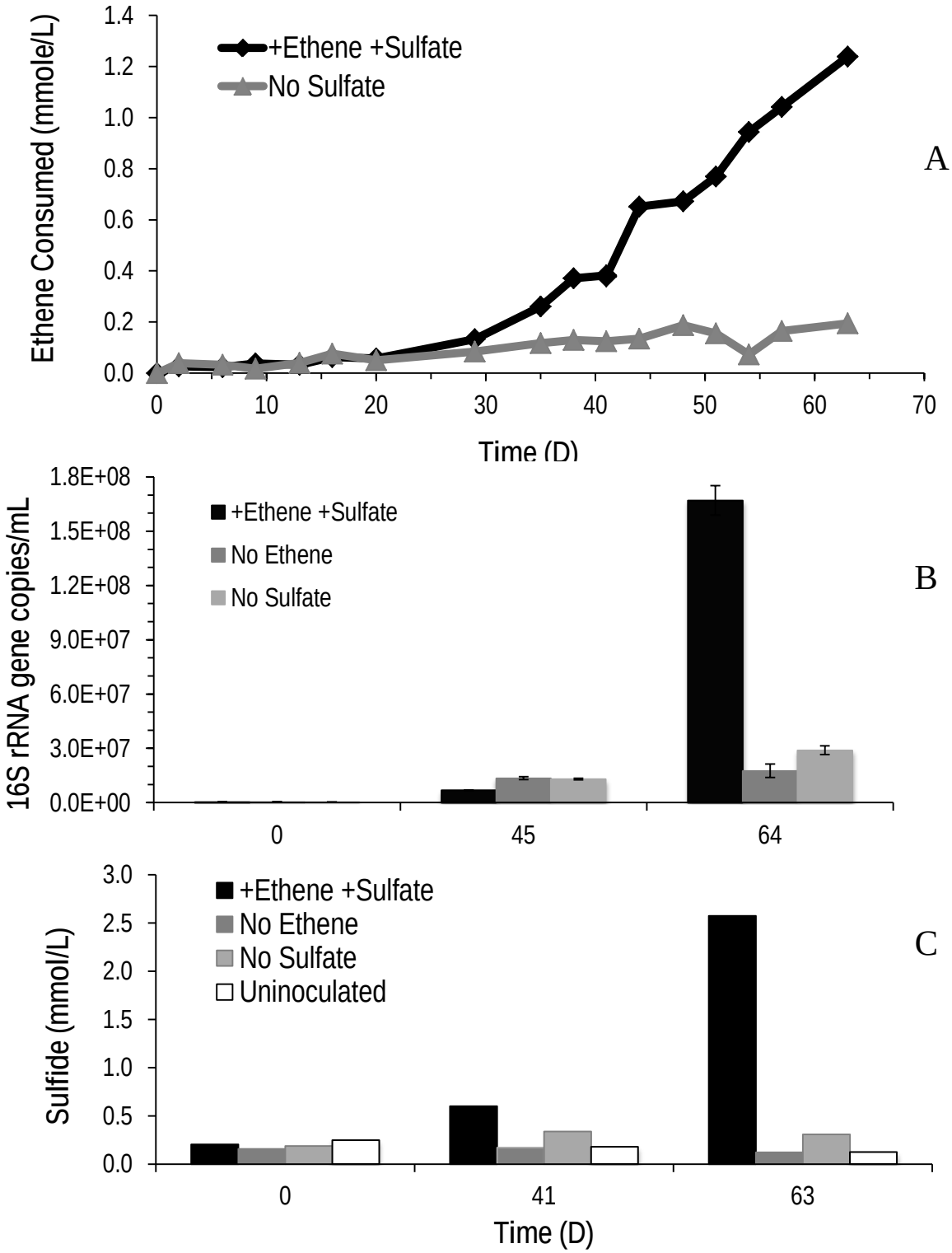


Figure 4.3. (A) Consumption of ethene normalized to abiotic control, (B) linked to an increase in 16S rRNA gene copies, and (C) production of sulfide in fourth generation transfers in comparison to vials without the addition of sulfate and without ethene, and abiotic control. Ethene results for individual tubes are presented in Figure S5; additional 16S rRNA and sulfide results are presented in Figure A-6.

The fifth and sixth generation generations of the sulfate-reducing culture continued to consume ethene (Figure A-7 and A-8, respectively). Two tubes from the fifth generation and three from the sixth were spiked with [^{14}C]ethene, along with abiotic controls (three tubes) and water controls (five tubes). After the labeled and unlabeled ethene was consumed (20-30 days) in the inoculated tubes, all were sacrificed to determine the fate of the ^{14}C (Figure 4.4). In the abiotic and water controls, more than 90% of the label was recovered as ethene with most of the remaining label as CO_2 (3-7%) or unaccounted for (1-5%). In comparison, the combined result for the fifth and sixth generation cultures showed 2.3%±1.5% of label remaining as ethene and 86.7%±3.3% as CO_2. Moreover, 4.4% of the label was found in the non-strippable residue fraction, representing ^{14}C that was either incorporated into cells or nonvolatile products, whereas no more than 0.5% of the label was found in this fraction in the control samples. No organic acids were detected in cultures receiving non-labeled ethene when analyzed by HPLC.

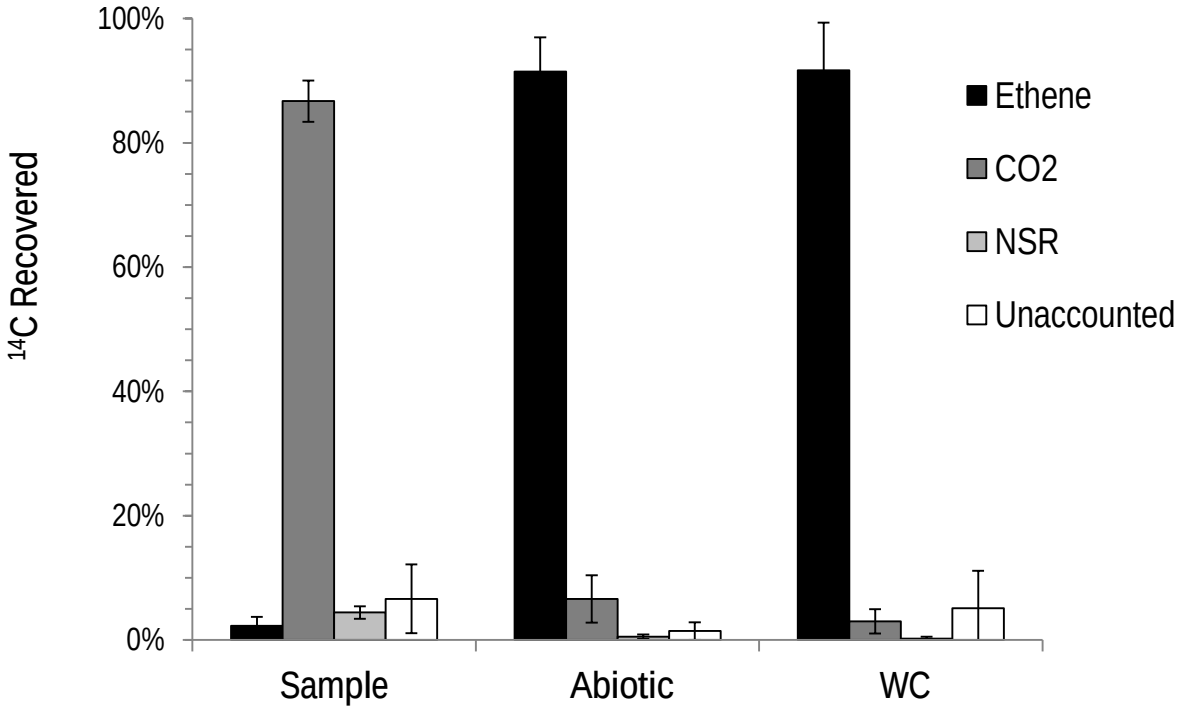


Figure 4.4. Distribution of ^{14}C from [^{14}C]ethene added to fifth and sixth generation enrichment cultures. NSR, non-strippable residue; WC, water control.

4.3 MICROBIOLOGICAL CHARACTERIZATION

Microscopic observation showed that the dominant morphotype was ovoid cells, 1-2 μm in diameter occurring in singlets or doublets. These ovoid cells were most often present within the amorphous iron sulfide particles usually used as a reductant for the culture (Figure 4.5A), although cells outside the particles were present. Two other cell morphologies were often viewed, a small motile curved rod similar to *Desulfovibrio* outside the FeS particles, and the other a slender rod shaped cell, which co-occurred within the iron sulfide particles. In successive transfers, the ovoid morphotype became a larger proportion of the total cells and transfers to medium without iron sulfide showed this dominance clearly. Figure 4.5B shows cells from a seventh generation culture growing in medium without FeS precipitates and reduced with 0.5 mM sodium sulfide, some of which contain granules of unknown composition. Growth in sulfide-reduced medium was variable.

A small 16S rRNA gene clone library was constructed using universal bacterial primers, from a sixth-generation transfer. Amplification with archaeal primers failed to produce a PCR product (data not shown). Of the 22 clones sequenced, 9 distinct sequences were present, and 4 sequences only occurred once. All of the sequences except two represent phylotypes that are considered anaerobic. Over half of the sequenced clones were nearly identical (≥98% identity) and were called MT6. They were most similar (91% Blast identity) to *Desulfovirga adipica* strain TsuA1 in a BLAST search using the 16S rRNA gene database for cultured organisms (Table 4.1, Figure 4.6). Strain TsuA1 was enriched from an anaerobic digestor with adipate provided as the electron donor and sulfate as the electron acceptor (Tanaka et al., 2000) and uses a variety of organic acids and alcohols. Other cultured members of the same clade (89% sequence identity) were members of the genus *Syntrophobacter*, known to couple propionate oxidation to sulfate reduction or to methanogens via syntrophic interactions. Indeed seven of the members of the MT6 clade were considered closer to *Syntrophobacter* (90% Blast identity) than *Desulfovirga* by the RDP classifier (rdp.cme.msu.edu/). The sequences in the nonredundant nucleic acid database sequences most closely resembling MT6 (Table 4.2) were from a methanogenic enrichment culture syntrophically converting limonene, a plant derived terpene containing a double bond, to methane (Rotaru et al., 2012). Sequences nearly identical to MT6 were found in clone libraries from earlier culture transfers and the Bellerophon chimera checker (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) did not classify it as a chimera, so it is unlikely that this sequence represents a chimera or other artifact.

The two next most numerous sequence types were distantly related to *Spirochaetes* cultured from termite guts. *Treponema primitia* grows acetogenically on H_2/CO_2, whereas *Treponema* sp. SPIT5 ferments saccharides to ethanol and CO_2. There were closer *Spirochaetae* relatives in the uncultured databases (Table 4.2), and several were derived from oil fields or hydrocarbon enrichments. *Rodanobacter*, a genus not known to oxidize hydrocarbons, is the only aerobic genus with 16S rRNA gene sequences most closely related to sequences in the clone library, and sequences related to known ethene oxidizers, such as *Mycobacterium*, *Xanthobacter*, and *Nocardioides*, were not found in the library.

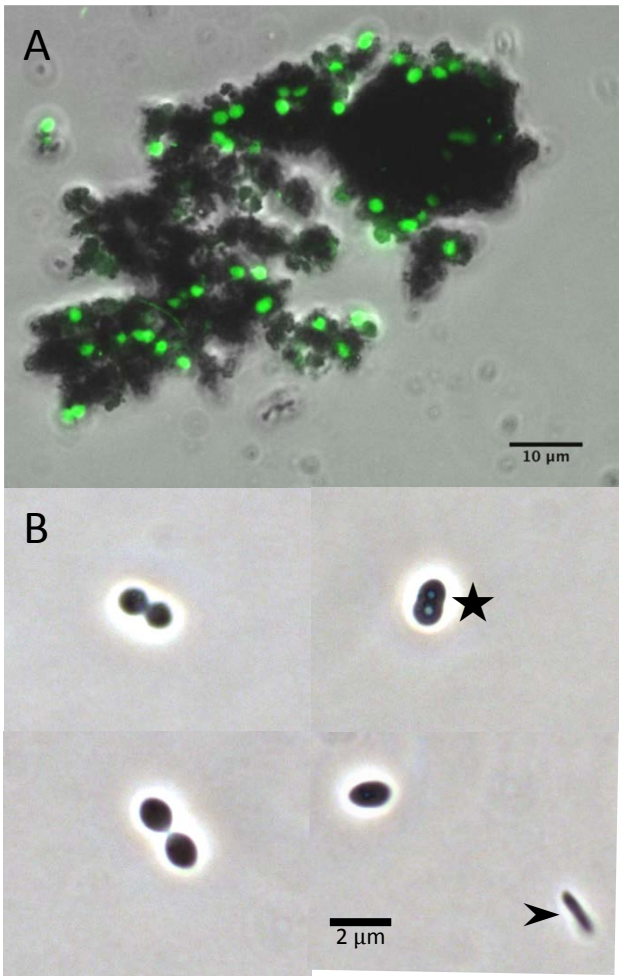


Figure 4.5. Photomicrographs of the ethene oxidizing sulfate-reducing enrichment cultures. (A) Composite phase-contrast epifluorescence micrograph of an FeS-reduced culture showing acridine orange-stained cells within an FeS particle. (B) Higher magnification micrographs of a sodium sulfide-reduced culture showing individual and dividing cells. (★), cells with granules; (➢) presumptive rod shaped contaminant. Photomicrographs were taken with a Nikon Eclipse E600 microscope equipped with an Infinity2 CCD camera. The drop used for a wet mount in (A) was stained with 2 µl of 0.01% acridine orange.

Table 4.1. Top Hits in a 16S rRNA gene clone library derived from a 6th generation ethene/sulfate culture to the 16S ribosomal RNA sequence (*Bacteria* and *Archaea*) BLAST database, and numbers of clones for each phylotype.

Best Hit for Sequenced Clone	GenBank Accession Number	Percent Identity	Number of Clones
Desulfovirga adipica strain TsuA1	NR_036764.1	91%	13/22
Treponema sp. SPIT5 strain SPIT5	NR_042486.1	86%	3/22
Treponema primitia ZAS-2 strain ZAS-2	NR_041714.1	86%	2/22
Levilinea saccharolytica strain KIBI-1	NR_040972.1	89%	1/22
Desulfovibrio mexicanus strain Lup1	NR_028776.1	98%	1/22
Rhodanobacter ginsenosidimutans strain CSC17Ta-90	NR_044467.1	96%	1/22
Rhodanobacter thiooxydans strain LCS2	NR_041565.1	98%	1/22

Table 4.2. Top hits in a 16S rRNA gene clone library derived from a 6th generation ethene/sulfate culture to the nonredundant nucleotide collection (nr/nt) BLAST database, and numbers of clones for each phylotype.

Best Hit for Sequenced Clone	GenBank Accession Number	Percent Identity	Number of Clones
Uncultured delta proteobacterium	FN646460.1	93%	13/22
Bacterial enrichment clone(*Spirochaetales*)	GU080088.1	99%	1/22
Uncultured *Spirochaetaceae* bacterium clone B6_81	HQ689205.1	99%	1/22
Uncultured bacterium clone LHJB-126 (*Spirochateales*)	JF741946.1	98%	2/22
Levilinea sp. P3M-1	JQ292916.1	97%	1/22
Uncultured bacterium clone D12_FB	EU981244.1	99%	1/22
Bacterium K19(2011)	HQ728406.1	96%	1/22
Uncultured bacterium SJA-102	AJ009481.1	99%	1/22
Rhodanobacter sp GR14-4	FJ821729.1	98%	1/22

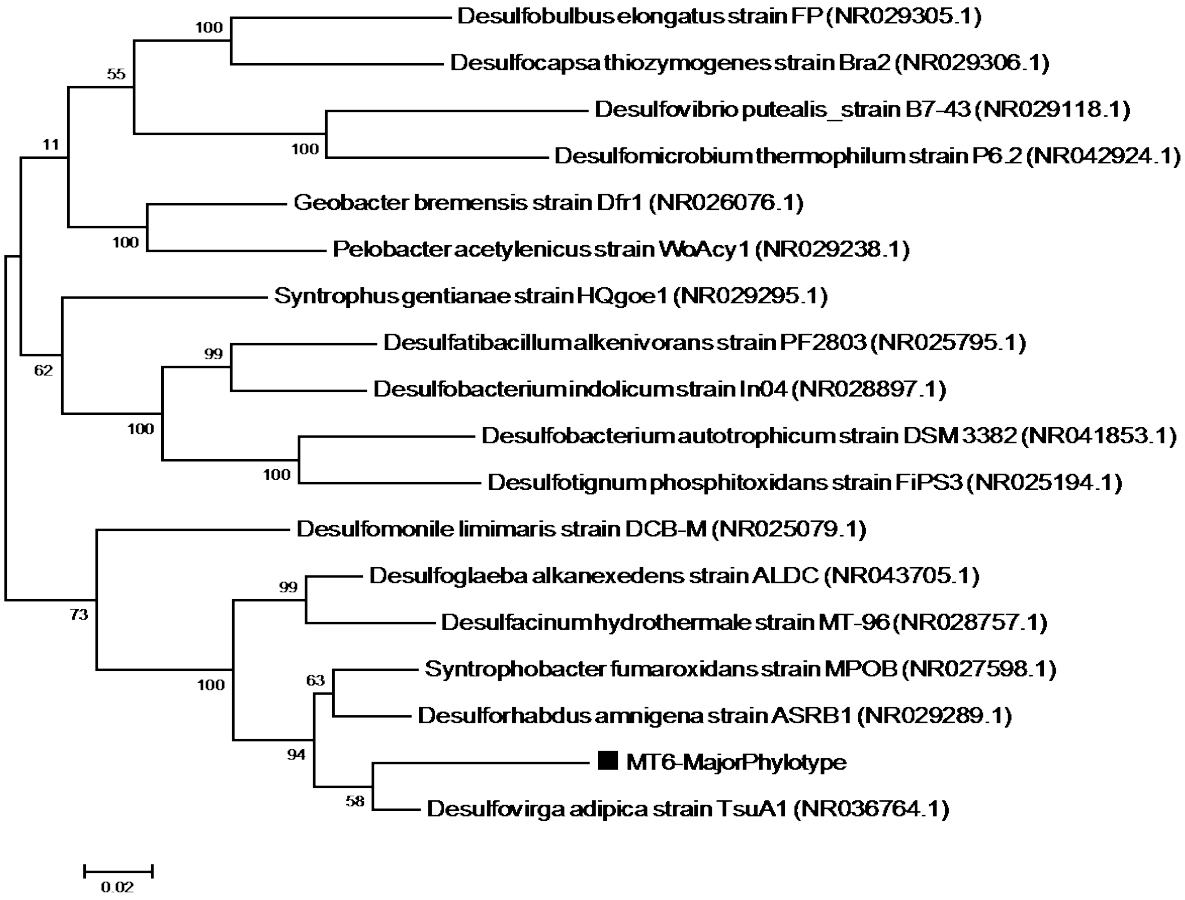


Figure 4.6. 16S rRNA Maximum Parsimony tree. (■) MT6 represents the most numerous phylotypes recovered from the clone library. Numbers at nodes are support of 100 bootstrap replications.

4.4 DISCUSSION

The evidence presented here demonstrates that ethene, considered recalcitrant under anaerobic conditions (Schink, 1985a; b), can be oxidized to CO_2 using sulfate as an electron acceptor, and that this couple supports microbial growth. The evidence supporting ethene oxidation to CO_2 includes the disappearance of considerable amounts of ethene without the detection of volatile organic products, such as methane or ethane, by gas chromatography, or of water soluble products like acetate by HPLC. Moreover, nearly all of the added [^{14}C]ethene was found in the $^{14}CO_2$ fraction after incubation with the culture, whereas in abiotic controls most of the radioactivity remained as ethene. That sulfate was the electron acceptor was demonstrated by the near stoichiometric accumulation of sulfide in the samples amended with ethene, but not in controls lacking ethene. The production of sulfide and lack of known aerobic ethene oxidizers as major components of the 16S rRNA gene clone library argue against the possibility that ethene oxidation was due to small amounts of oxygen entering the microcosms and cultures (Gossett, 2010).

The estimated free energy for ethene oxidation coupled to sulfate reduction under culture conditions is only a few kJ different from that in Equation 1.5, -136.3 kJ/rxn, indicating that energy can be conserved from this reaction thereby supporting microbial growth. These cultures were incubated in medium in which, other than trace amounts of vitamins, ethene served as the sole electron donor and organic carbon source, and sulfate was the only added potential electron acceptor other than CO_2. These cultures were transferred multiple times, a condition that requires growth of the biocatalyst. Ethene utilization rates increased over time in the cultures, also consistent with microbial growth. Moreover, microbes were readily apparent in cultures that had consumed ethene, and bacterial numbers, as estimated by qPCR, increased greatly relative to cultures lacking ethene or sulfate. Thus it is clear that the ethene/sulfate couple supported growth of microorganisms in these cultures.

The most numerous phylotype found in the 16S rRNA gene clone library, called MT6, is a member of the *Deltaproteobacteria*, most closely related to *Desulfovirga adipica* and several *Syntrophobacter* spp., organisms that carry out reactions with relatively low thermodynamic yields, and is somewhat more distantly related to *Desulfoglaeba alkanexedens*, a hydrocarbon utilizer. The MT6 16S rRNA gene is ≤91% identical with those from cultured organisms, and had ≤93% identity with the entire NCBI nr database, demonstrating how unique this phylotype is. While this distance precludes physiological conclusions based on phylotype, it is a reasonable candidate for an organism responsible for ethene oxidation and sulfate reduction.

The dominant morphotype in the cultures was a relatively large egg shaped cell, and it is tempting to conclude that the MT6 phylotype has this morphology which many organisms with related sequences share, but further studies are necessary to demonstrate this proposition. The second most abundant phylotypes in our small clone library belonged to the phylum *Spirochaetes*, a group not known to use hydrocarbons. It is difficult to explain their presence except to speculate that, being distantly related to acetogenic spirochetes (86% identity), some may have grown on CO_2 and residual H_2 left after flushing the culture vials with N_2/CO_2. Also intriguing is that many of the related sequences (98-99% identity) in the nonredundant NCBI database are derived from environments or cultures where hydrocarbons were present.

Ethene produced by reductive dechlorination of chloroethenes in anaerobic zones of contaminated sites is often stable, but under some circumstances can be reduced to ethane, a process possibly associated with methanogenesis (de Bruin et al., 1992; Koene-Cottaar and Schraa, 1998; Mundle et al., 2012). The reasons why ethane is produced by some samples and not others are not understood. Bradley and Chapelle (2002) found that methanogenic sediments from a lake receiving groundwater containing chloroethenes produced methane and converted added [^{14}C]ethene to ethane. However, when those sediments were amended with sulfate, methanogenesis was inhibited and [^{14}C]ethene was oxidized to $^{14}CO_2$, implicating sulfate reducing microorganisms in ethene oxidation. The studies presented here support and expand upon those results. In the previous study (Bradley and Chapelle, 2002), a single dose of 0.1 mmol/L ethene was consumed in 166 days. While there was a lag of about 40 days before $^{14}CO_2$ was detected, it was not clear that the rate increased over time. In this study, multiple doses of 0.4-1.5 mmol/L were consumed in microcosms and cultures, with rates increasing over time, indicative of growth.

In light of these and previous results, the possibility of ethene oxidation to CO_2 in sulfate reducing zones should be considered. If ethene is reduced to ethane *in situ*, the ethane can be readily detected by gas chromatography and contribute to mass balance determinations. However, if ethene is oxidized, it joins the large CO_2 pool and cannot be accounted for in the mass balance, leading to a deficit, as has been found in some site studies (Bradley, 2003). Presently, there is no way to predict whether this reaction is occurring other than microcosm studies. It is possible that anaerobic ethene oxidation will have a stable isotope fractionation signature as does reduction to ethane (Mundle et al., 2012). It is also possible that the unique bacterial phylotype we have found associated with this reaction can serve as a biomarker for it, but considerably more study will be needed to support or refute this hypothesis.

5.0 RESULTS AND DISCUSSION: NEGATIVE OR EQUIVOCAL OUTCOMES

Results for each set of microcosms are presented in the order in which they appear in Table 2.2, i.e., by site number (1-10), set number (I-IV) and subset letter (A-D). If enrichments were prepared from a set of microcosms, those results are presented following the microcosms for that site. Figures that show results for the volatile organic compounds (VC, ethene, ethane, methane, and cDCE) are presented in terms of µmoles per bottle. This allows for a direct inspection of the stoichiometry of reduction, since one mole of daughter product is expected per mole of parent compound, unless a process other than reduction is responsible for the decrease. Amounts per bottle may be related to the aqueous phase concentration by using equation 2.1.

5.1 SITE #1

Ten subsets of microcosms were prepared with groundwater, and in some cases soil, from Site #1. Results for each subset are presented sequentially as they appear in Table 2.2.

5.1.1 Site #1, Set I-A

This set of microcosms was prepared with 100 mL of first flush groundwater from well MW-6. The groundwater contained significant amounts of TCE, cDCE and DCM, to which VC was also added. Treatments were prepared without amendments as well as with five electron acceptors (Fe(III), Fe(III)-EDTA, Mn(IV), AQDS, and SO_4^{2-}); 10 replicates were used for each treatment. Resazurin was added to the groundwater to serve as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. The COD of groundwater from well MW-6 was 174 mg/L and the DOC was 33 mg/L, suggesting that electron donor was available for reductive processes.

The bottles were monitored for nearly 800 days. Average results for VC indicated no appreciable decrease in any of the treatments, either via reduction or oxidation (Figure 5.1). There was evidence of TCE reduction to cDCE, but no significant reductive dechlorination of cDCE to VC or VC to ethene. There was also no evidence of DCM biodegradation or methanogenesis. Accumulation of Fe(II) and Mn(II) and consumption of sulfate indicated these added TEAs were reduced; AQDS reduction was no monitored.

5.1.2 Site #1, Set I-B

This set of microcosms was prepared with 100 mL of first flush groundwater from Site #1, well #7. No significant amounts of VOCs were present in the groundwater, including VC, which was added (Table 2.2). Treatments were prepared without amendments as well as with the same electron acceptors as with Set I-A. Resazurin was added to serve as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. The COD of groundwater from well MW-7 was 131 mg/L and the DOC was 7 mg/L, suggesting that electron donor was available for reductive processes, although at a lower concentration than in water from well MW_6.

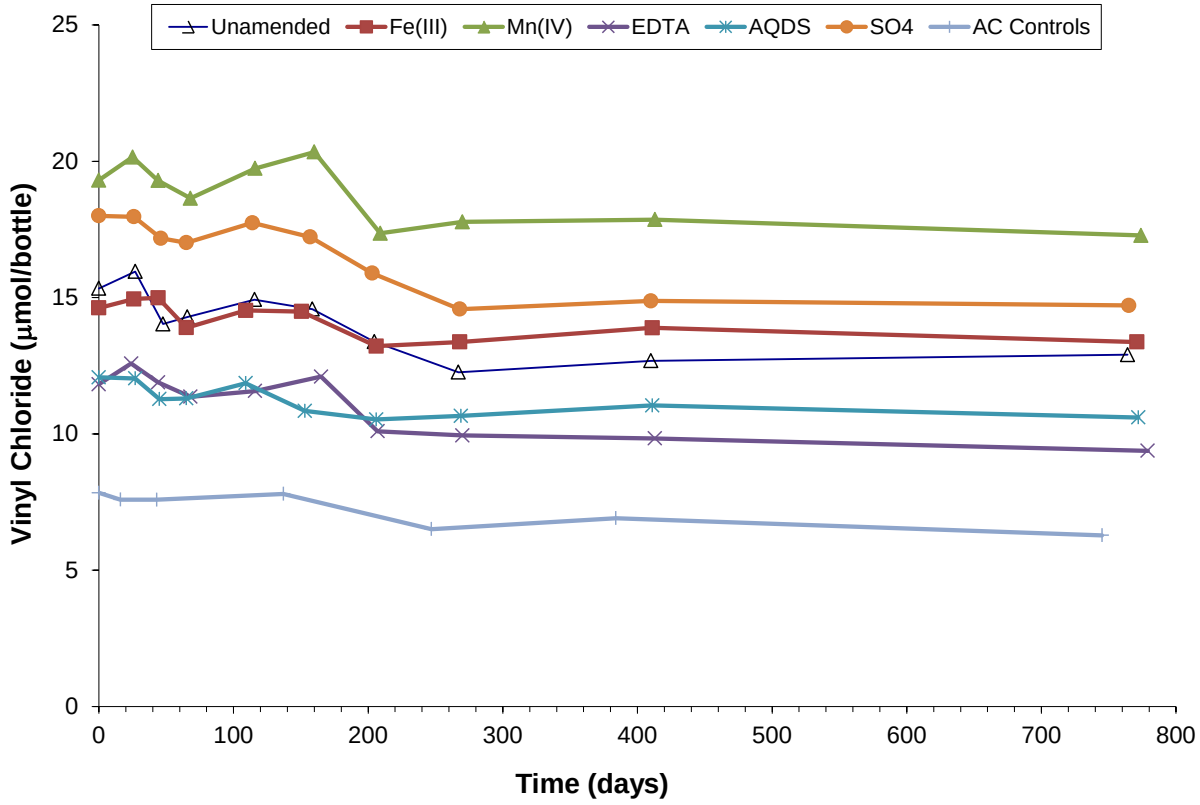


Figure 5.1. Average VC levels in the microcosms for Site #1, Set I-A.

The bottles were monitored for approximately 800 to 1100 days. The predominant activity observed was reductive dechlorination of VC to ethene; there was no indication of anaerobic oxidation. Figure 5.2 shows the cumulative amounts of VC reduced to ethene. When VC was consumed, one to five repeat additions of VC were made over the incubation period. The decreased rate of VC reduction over time was likely a consequence of depletion of electron donor. In most cases addition of TEA reduced the rate of VC reduction, with the exception of Fe(III) addition. Relative to the amount of electron donor used for dechlorination, a significant level of methane production occurred in nearly all of the live bottles (ca. 0.5-1.5 mmol/bottle).

Average losses from the autoclaved controls were 12% for VC and 1% for ethene. These results confirmed that grey butyl rubber septa were effective in preventing diffusive losses of ethene and VC during extended periods of incubation.

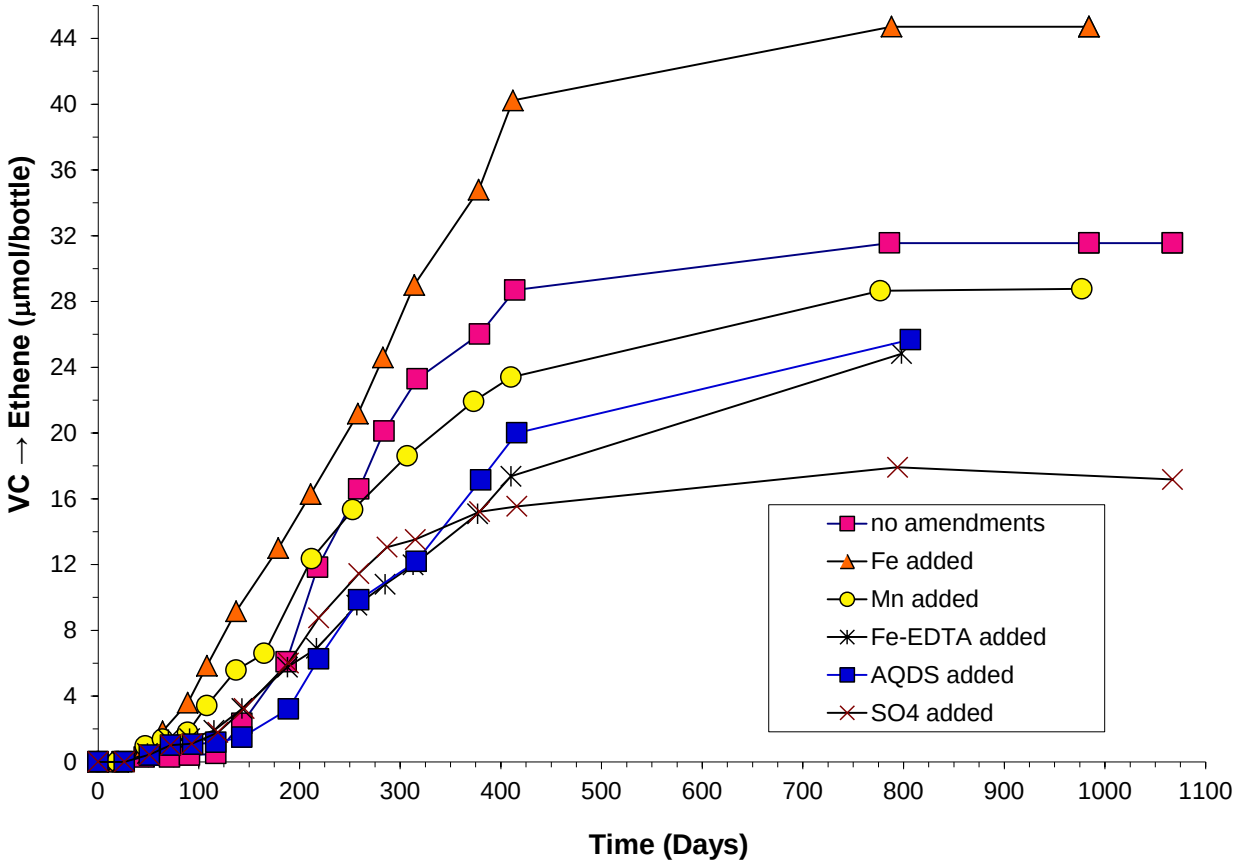


Figure 5.2. Cumulative reduction of VC to ethene in the Site #1, Set I-B microcosms.

5.1.3 Site #1, Set II-A

This set of microcosms was prepared with 20 g of sediment and 50 mL of first flush groundwater from Site #1, well MW-6. The groundwater contained significant amounts of cDCE and DCM, to which VC was also added. Sediment cores from the site arrived approximately five weeks after the groundwater. During this period, while the groundwater was stored at 4°C, most of the volatile compounds in the groundwater diffused from the plastic storage containers, resulting in much lower initial concentrations. Only a trace amount of TCE remained in the groundwater (0.02 mg/L), while cDCE and DCM levels were reduced by 95% and 87% to 0.5 mg/L and 7.3 mg/L, respectively. Only unamended microcosms were prepared for this subset, due to the limited availability of soil from this part of Site #1 and initial results of relatively inactive Site #1 Set I-A microcosms (which were prepared with the same groundwater, i.e., from well #6). Resazurin was added to the groundwater to serve as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. The initial COD of groundwater from MW-#6 was 174 mg/L and the DOC at 33 mg/L, suggesting that electron donor was available for reductive processes.

The bottles were monitored for 380 days. No significant decrease in VC occurred in nine of the 12 bottles; in the other three, VC was stoichiometrically dechlorinated to ethene. There was no indication of anaerobic oxidation in any of these microcosms. As with the Set I-A bottles, there was virtually no methane production. The most notable behavior of these microcosms was the complete consumption of DCM within the first three months of incubation, in all of the bottles, without accumulation of chloromethane or methane. This indicated the presence of microbes capable of fermenting DCM. Samples from these microcosms have subsequently been used to develop an enrichment culture that grows anaerobically on DCM as the sole carbon and energy source.

5.1.4 Site #1, Set II-B

This set of microcosms was prepared with 20 g of sediment and 50 mL of first flush groundwater from Site #1, well MW-7. No significant amounts of VOCs were present in the groundwater, thus VC was added. Treatments were prepared without amendments as well as with the five electron acceptors described above. Resazurin was added to the groundwater to serve as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. The COD of groundwater from well MW-7 was 131 mg/L, suggesting that electron donor was available for reductive processes.

The bottles were monitored for approximately 780 to 930 days. The predominant activity observed was reductive dechlorination of VC to ethene; there was no indication of anaerobic oxidation. Figure 5.3 shows the cumulative amounts of VC reduced to ethene. When VC was consumed, repeat additions of VC were made over the incubation period. The decreased rate of VC reduction over time was likely a consequence of depletion of electron donor and/or a reduced frequency of monitoring. Addition of TEA had mixed effects; Fe(III), Fe(III)-EDTA, and sulfate enhanced VC reduction; Mn(IV) and especially AQDS inhibited VC reduction. Methane output in all of the treatments was comparable to the levels observed with Set I-B.

5.1.5 Site #1, Sets III-A and III-B

Microcosms were prepared with 100 mL of first flush groundwater from Site #1, wells #RW-08 and #RW-10; amendments were not added. No significant amounts of VOCs were present in the groundwater, thus VC was added. Resazurin was added to the groundwater to serve as a redox indicator. Based on the change in the color of the groundwater from pink to clear (shortly after all of the bottles were started for RW-08; 141-231 days for RW-10), the redox level was below -110 mV for the majority of the incubation period. As shown in Figure 5.4, none of these microcosms consumed significant amounts of VC, following 1410 days of incubation. There was also no significant level of methane accumulation. The COD of groundwater was 106 mg/L in RW-08 and 77 mg/L in RW-10, suggesting that electron donor was available for reductive processes, yet reductive dechlorination did not occur. Fe(II) was 0.95 mg/L in RW-08 and 0.08 mg/L in RW-10 groundwater; Mn(II) and sulfate were not measured.

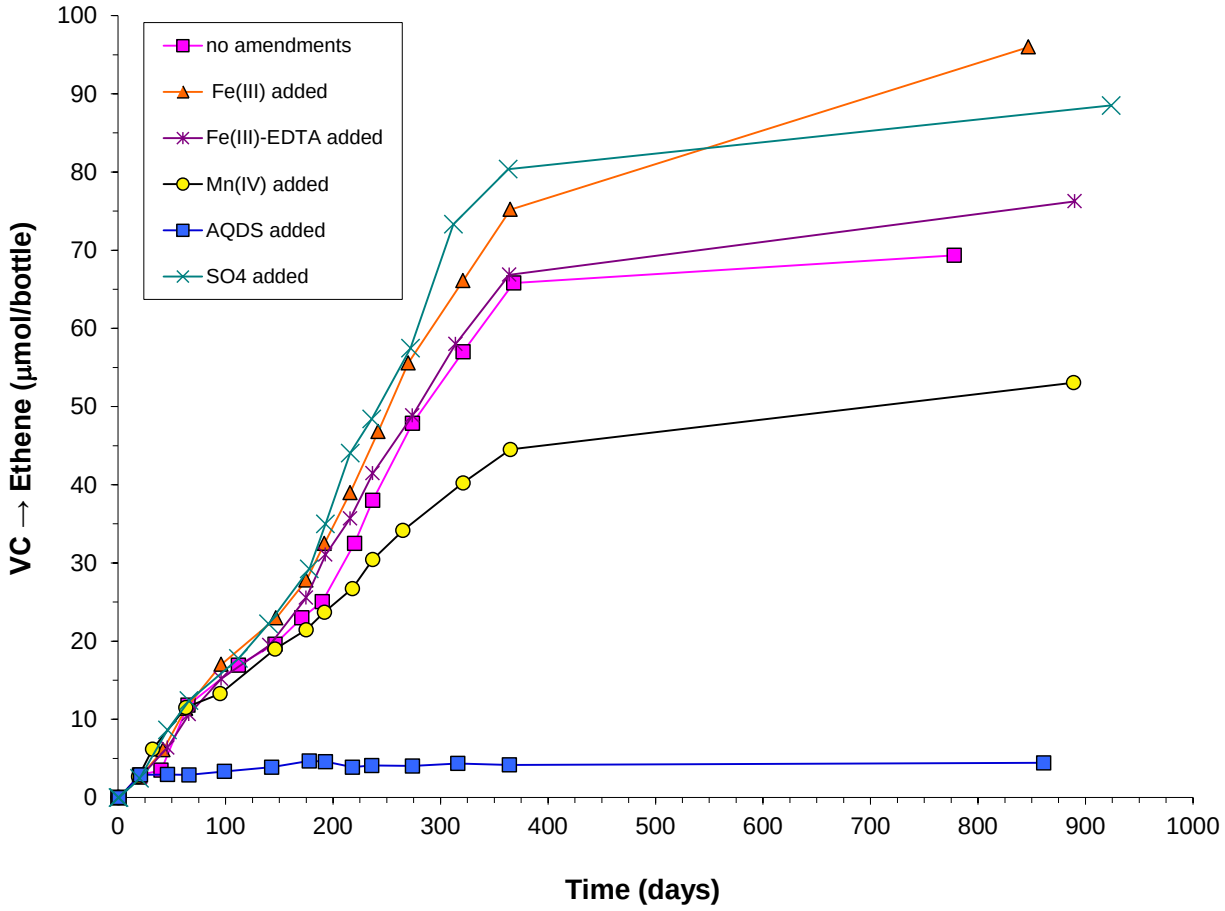


Figure 5.3. Cumulative reduction of VC to ethene in the Site #1, Set II-B microcosms.

5.1.6 Site #1, Set IV-A

This set of microcosms was prepared with 20 g of sediment (core #MW-2118-72 #2219) and 50 mL of first flush groundwater from Site #1, well #2114. Groundwater initially contained approximately 0.55 mg/L of TCE (± 0.07 mg/L) and only trace levels of cDCE and VC. A total of 15 live microcosms and three autoclaved controls were prepared in the anaerobic chamber without amendments and without subsequent purging. After seven days of incubation, 1.1 µmol/bottle (± 0.1 µmol/bottle) of neat VC gas was added to all of the microcosms, and approximately 0.1 µmol/bottle of [^{14}C]VC was added to five live microcosms (IA-1 through IA-5) and one autoclaved control (IA AC 1). Resazurin was added to the groundwater as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period.

Microcosms in this set were incubated in the anaerobic chamber for 617 days. There was no evidence of VC biodegradation, either by reduction or oxidation (data not shown). VC levels in the live bottles were no different than in the autoclaved controls. Consequently, no attempt was

made to evaluate the distribution of ^{14}C in the bottles that received [^{14}C]VC. There was also no biodegradation of TCE in the live bottles. The hydrogen initially present in the headspace (ca. 4.5 μmol/bottle, since the bottles were not purged after preparation) was below detection in the live bottles within 24 days of incubation. There was no significant level of methane production.

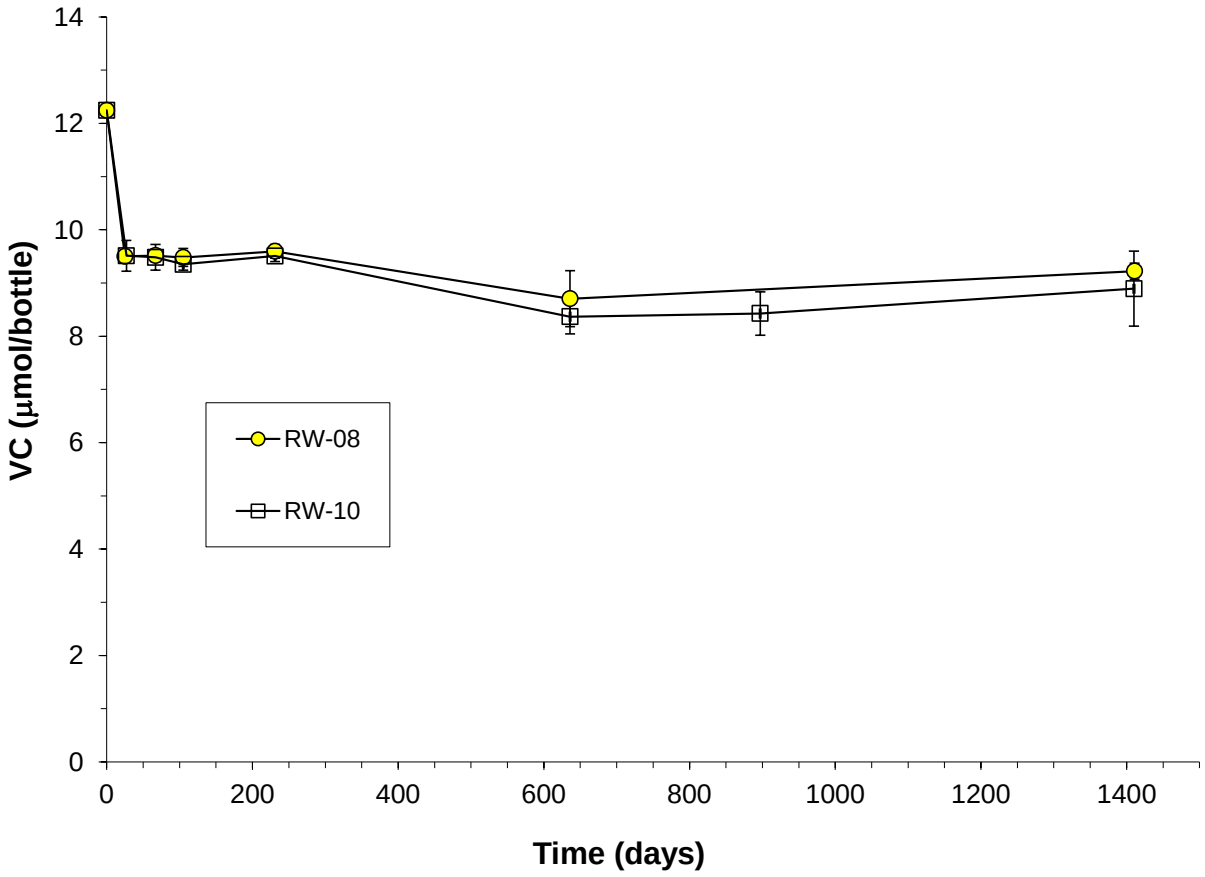


Figure 5.4. Results for VC in the Site #1, Set III-A (RW-08) and III-B (RW-10) microcosms.

5.1.7 Site #1, Set IV-B

This set of microcosms was prepared with 20 g of sediment (core #MW-2118-72 #2219) and 50 mL of first flush groundwater from Site #1, well #2114. Groundwater initially contained approximately 0.55 mg/L of TCE (± 0.07 mg/L) and only trace levels of cDCE and VC. All microcosms were prepared in the anaerobic chamber without amendments, identically to Set IV-A. However, with Set IV-B, all bottles were purged with oxygen-free N_2 to remove residual hydrogen, which was present in the headspace of the bottles from the atmosphere of the anaerobic chamber. The bottles were purged for 10 min for two consecutive days. After six days of incubation, 1.0 μmol/bottle (± 0.0 μmol/bottle) of neat VC gas was added to all of the microcosms, and approximately 0.2 μmol/bottle of [^{14}C]VC was added to five live microcosms (IB 1 through IB 5) and one autoclaved control (IB AC 1). Resazurin was not added to these microcosms and they were incubated outside of the anaerobic chamber.

Twelve of the microcosms in this set were incubated in the anaerobic chamber for 617 days; three were incubated for 1245 days. There was no evidence of VC biodegradation, either by reduction or oxidation (data not shown). VC levels in the live bottles were no different than in the autoclaved controls (incubated for 1245 days). Consequently, no attempt was made to evaluate the distribution of ^{14}C in the bottles that received [^{14}C]VC. There was also no biodegradation of TCE in the live bottles. Hydrogen was purged from the headspace immediately after set-up and was not detected on day three (after purging had been completed) or on days 24 and 50. There was no significant level of methane production throughout the incubation period.

5.1.8 Site #1, Set IV-C

This set of microcosms was prepared with 20 g of sediment (core #MW-2113-48 #2151) and 50 mL of first flush groundwater from Site #1, well #2114. The sediment came from a different location than the samples used in Sets I-A and I-B. Groundwater initially contained approximately 0.55 mg/L of TCE (± 0.07 mg/L) and only trace levels of cDCE and VC. All microcosms were prepared in the anaerobic chamber without amendments, identically to Set IV-A (i.e., the bottles were not purged with N_2). After six days of incubation, 1.1 μmol/bottle (± 0.1 μmol/bottle) of neat VC gas was added to all of the microcosms, and approximately 0.1 μmol/bottle of [^{14}C]VC was added to five live (IIA 1 through IIA 5) microcosms and one autoclaved control (IIA AC 1). Resazurin was added as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. Microcosms in this set were incubated in the anaerobic chamber.

Microcosms in this set were incubated in the anaerobic chamber for 648 days. There was no evidence of VC biodegradation, either by reduction or oxidation (data not shown). VC levels in the live bottles were no different than in the autoclaved controls. Consequently, no attempt was made to evaluate the distribution of ^{14}C in the bottles that received [^{14}C]VC. There was also no activity on TCE in the live bottles. The hydrogen initially present in the headspace (ca. 6.4 μmol/bottle , since the bottles were not purged after preparation) was below detection in most of the live bottles within 92 days of incubation. There was no significant level of methane production.

5.1.9 Site #1, Set IV-D

This set of microcosms was prepared with 20 g of sediment (core #MW-2113-48 #2151) and 50 mL of first flush groundwater from Site #1, well #2114. Groundwater initially contained approximately 0.55 mg/L of TCE (±0.07 mg/L) and only trace levels of cDCE and VC. All microcosms were prepared in the anaerobic chamber without amendments, similarly to Set IV-C. However, with this subset, all of the bottles were purged with oxygen-free N_2 to remove residual hydrogen, which was present in the headspace of the bottles from the atmosphere of the anaerobic chamber. After six days of incubation, 1.0 μmol/bottle of neat VC gas was added to all microcosms, and approximately 0.1 μmol/bottle of [^{14}C]VC was added to five live microcosms (IIB 1 through IIB 5) and one autoclaved control (IIB AC 1). Resazurin was not added to these microcosms and they were incubated outside of the anaerobic chamber.

Twelve of the microcosms in this set were incubated in the anaerobic chamber for 1251 days. There was no evidence of VC biodegradation, either by reduction or oxidation (data not shown). VC levels in the live bottles were no different than in the autoclaved controls (incubated for 1245 days). Consequently, no attempt was made to evaluate the distribution of ^{14}C in the bottles that received [^{14}C]VC. There was also no biodegradation of TCE in the live bottles. Hydrogen was purged from the headspace immediately after set-up and was not detected on day three (after purging had been completed). There was no significant level of methane production throughout the incubation period.

5.2 SITE #2

5.2.1 Site #2, Set I-A

A total of 15 microcosms were prepared with 100 mL of first flush groundwater from Site #2, well #92. No significant amounts of VOCs were present in the groundwater, thus VC was added. Resazurin was added to the groundwater to serve as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. The microcosms in this set were incubated under anaerobic conditions for 604 d. During this interval, there was no evidence of VC biodegradation, either by oxidation or reduction. A modest level of methane production (ca. 5-25 µmol/bottle) occurred in all but two of the bottles. Incubation of three of 15 bottles continued under anaerobic conditions though 1461 days, with no change in behavior.

The other 12 bottles were used to evaluate the response to a change from anaerobic to aerobic conditions, beginning on day 604. Oxygen was added (0.25-1.5 mL per addition) and monitored; more was added as it was depleted. It became apparent that the groundwater contained biodegradable organic matter that exerted an oxygen demand. At least 70 µmol/bottle of oxygen was added before a residual amount persisted. Nevertheless, a significant decrease in VC occurred in only two of the bottles; results for one of the bottles are shown in Figure 5.5. The decrease in VC coincided with a decrease in methane; as methane levels decreased, the rate of VC consumption slowed. This suggested cometabolic degradation of VC by methanotrophs.

5.2.2 Site #2, Set I-B

Microcosms were prepared with 100 mL of first flush groundwater from Site #2, well #93. No significant amounts of VOCs were present in the groundwater, thus VC was added. Resazurin was added to the groundwater to serve as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. Microcosms in this set were incubated in the anaerobic chamber for 1461 days. Approximately 80% of the VC dechlorinated to ethene during this time period; there was no evidence of anaerobic oxidation (data not shown). The COD of groundwater from well #93 was 108 mg/L, suggesting that electron donor was available for reductive processes. Methane output was approximately twice as high as in Set I-A.

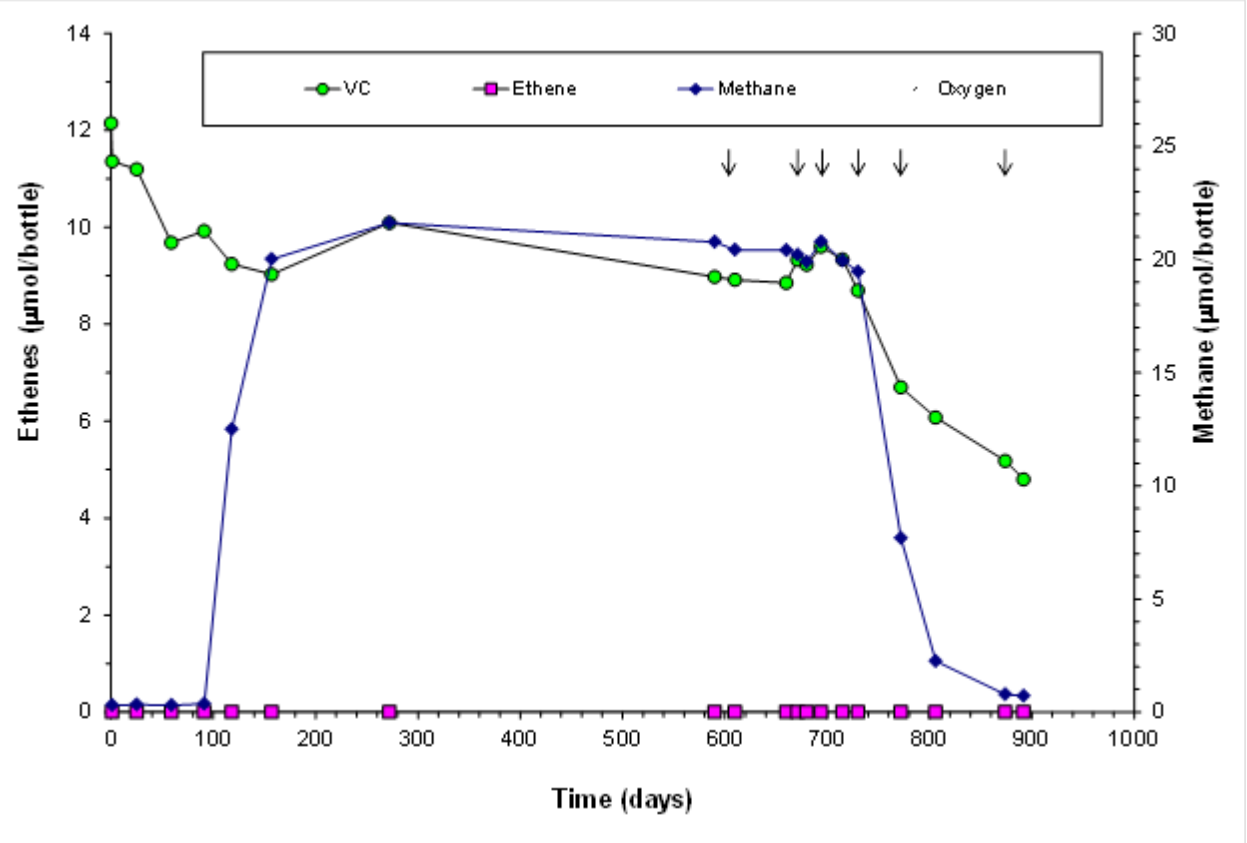


Figure 5.5. Results for a Site #2, Set I-A microcosm that was switched from anaerobic to aerobic conditions on day 604 by adding oxygen; each arrow represent 0.1 mL of oxygen (ca. 4 µmol).

5.3 SITE #3

This set of microcosms was prepared with 20 g of sediment and 50 mL of first flush groundwater from Site #3. No significant amounts of VOCs were present in the groundwater, thus VC was added. Treatments were prepared without amendments (10 bottles) as well as with five electron acceptors (Fe(III), Fe(III)-EDTA, Mn(IV), AQDS, and SO_4^{2-}; 5 bottles for each TEA) and triplicate water controls. Resazurin was added to the groundwater as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. The COD of the groundwater was 119 mg/L, suggesting that electron donor was available for reductive processes. The bottles were incubated for 1076-1120 days.

Ten unamended microcosms were prepared. Neat VC was added to all ten and [^{14}C]VC was added to five. Reductive dechlorination began immediately, albeit at a slower rate relative to many of the Site #1 Set II-B microcosms. An average of 341 days was required to biodegrade

the initial 12.9 µmol/bottle of VC. In general, the second addition of VC was consumed at a similar or slower rate. The average cumulative amount of VC reduced to ethene is shown in Figure 5.6. The reduction of VC to ethene was close to stoichiometric; there was no evidence in support of anaerobic oxidation of VC. Methane output averaged 86 µmol/bottle. COD levels in the groundwater were measured on day 431 and averaged 45 mg/L (± 15 mg/L). DOC was measured on day 272 and averaged 223 mg/L (± 41 mg/L).

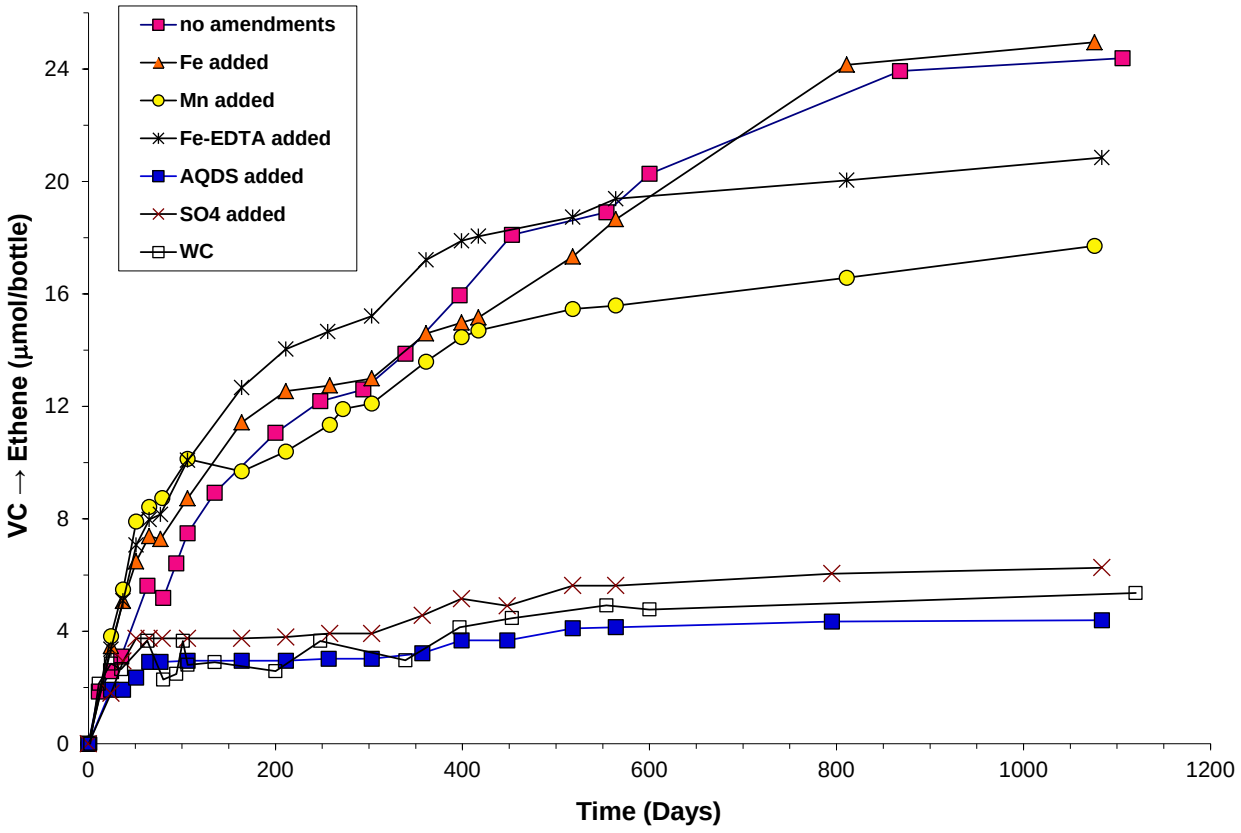


Figure 5.6. Cumulative reduction of VC to ethene in the Site #3 microcosms.

In the five unamended bottles that initially received [^{14}C]VC, a second addition of [^{14}C]VC was made when the bottles were respiked with neat VC gas on day 308. On three occasions (days 342, 469, and 574, or 608), the distribution of ^{14}C was determined based on analysis of headspace samples. As expected from the GC headspace monitoring results, the primary daughter product of [^{14}C]VC biodegradation was [^{14}C]ethene, which is consistent with the results from routine GC. $^{14}CH_4$ was never above 1% and [^{14}C]ethane was never above 2% of the ^{14}C added. During the third round of testing for ^{14}C (on day 574 or 608), a liquid sample was removed along with the headspace sample in order to measure $^{14}CO_2$ and [^{14}C]NSR. Both categories represented less than 1% of the ^{14}C added, indicating that no mineralization of VC had occurred up to that point in time.

In the treatments with TEA added, the predominant biotic activity was also reductive dechlorination of VC to ethene, as shown in Figure 5.6. There was no indication of any significant level of VC anaerobic bio-oxidation (i.e., consumption of VC without accumulation of ethene and/or ethane). Addition AQDS and sulfate inhibited reductive dechlorination; VC losses from these treatments was similar to the water controls. Fe(II) was detected in the Fe(III)-EDTA amended treatment but not in the treatment with Fe(III) added. Mn(II) was detected, although at levels well below the amount of Mn(IV) added. Approximately 20% of the sulfate available was consumed. Methane output in the Fe(III), Fe(III)-EDTA and Mn(IV)-amended treatments was similar to the unamended bottles, but was completely inhibited in the AQDS and sulfate-amended treatments.

5.4 SITE #4

Four subsets of microcosms were prepared with groundwater and sediment from Site #4. Two types of soil cores were used along with one type of groundwater (from a non-specified well). Two methods of preparation were used, resulting in four subsets of bottles (A, B, C and D). For subsets A and C, the headspace of the microcosms was not purged, while for B and D, the headspaces were purged to remove hydrogen from the anaerobic chamber's atmosphere. For each subset, 15 live bottles were prepared without amendments, along with three autoclaved controls, for a total of 18 bottles per subset.

Measurements for Fe(II) and Mn(II) in the groundwater were made, but neither was detected. Sulfate was initially measured at 2,341 mg/L (± 281 mg/L), which was comparatively high with respect to other sites. The COD and DOC of the groundwater was not tested.

5.4.1 Site #4, Set I-A

Site #4 Set I-A microcosms were prepared with 20 g of soil (ATP-6) and 50 mL of first-flush groundwater from a nearby well. Only trace amounts of cDCE and VC were present in the groundwater, thus VC was added. Eighteen microcosms were prepared in the anaerobic chamber without amendments and without subsequent purging; three of microcosms were used as autoclaved controls. After 13-14 days of incubation, 1.1 μmol/bottle (±0.2 μmol/bottle) of neat VC gas was added to all microcosms, and approximately 0.2 μmol/bottle of [^{14}C]VC was added to five live microcosms and one autoclaved control. Resazurin was added to serve as a redox indicator. Based on the change in the color of the groundwater from pink to clear shortly after all of the live bottles were started, the redox level was below -110 mV for the majority of the incubation period.

The microcosms were incubated for 543 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the six bottles that received [^{14}C]VC. Methane output in the live microcosms averaged 9 μmol/bottle.

5.4.2 Site #4, Set I-B

Site #4 Set I-B microcosms were prepared in the same manner as Set I-A, with the following key differences. With Set I-B, the bottles were purged with oxygen-free N_2 to remove residual hydrogen, which was present in the headspace of the bottles from the atmosphere of the anaerobic chamber. The bottles were purged for 10 min with high oxygen-free N_2 for two consecutive days. After 13-14 days of incubation, 1.1 μmol/bottle (± 0.1 μmol/bottle) of neat VC gas was added to all of the microcosms, and approximately 0.2 μmol/bottle of [^{14}C]VC was added to five live microcosms and one autoclaved control. Resazurin was not added to these microcosms and they were incubated outside of the anaerobic chamber.

The microcosms were incubated for 1259 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the six bottles that received [^{14}C]VC. Methane output in the live microcosms averaged 0.91 μmol/bottle.

5.4.3 Site #4, Set I-C

Site #4 Set I-C microcosms were prepared in the same manner as the Set I-A bottles, except that a difference source of soil was used (ATP-7). Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. The microcosms were incubated for 550 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the six bottles that received [^{14}C]VC. There was no methane output in any of the live microcosms.

5.4.4 Site #4, Set I-D

Site #4 Set I-D microcosms were prepared in the same manner as the Set I-B bottles, except that a difference source of soil was used (ATP-7). The microcosms were incubated for 1259 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the six bottles that received [^{14}C]VC. Methane output in the live microcosms averaged 0.18 μmol/bottle.

5.5 SITE #5

Four subsets were prepared for Site #5 microcosms, using two different types of soil and two types of preparation methods. Preparation of subsets B and D included purging of the headspaces to remove hydrogen; the other two subsets (A and C) were not purged. Subsets A and B were prepared with one type of sediment and C and D with another type. Enough sediment was available to prepare only six bottles per subset, four of which were live and two of which were

autoclaved, or 24 bottles in total. For Site #5, all of the microcosms received 20 g of sediment + 6 mL of autoclaved DDI water; groundwater was not available.

5.5.1 Site #5, Set I-A

Microcosms were prepared in the anaerobic chamber without amendments and without subsequent purging, using Kings Bay outcrop sediment. After nine days of incubation, 1.0 μmol/bottle (± 0.0 μmol/bottle) of neat VC gas was added to all of the microcosms, and approximately 0.2 μmol/bottle of [^{14}C]VC was added to two of the live microcosms and one of the autoclaved controls. Resazurin was added to the DDI water to serve as a redox indicator. However, the water in the microcosms was too dark to see any color changes from the resazurin. Microcosms in this subset were incubated in the anaerobic chamber for 538 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the six bottles that received [^{14}C]VC. Methane output in the live microcosms was below 1 μmol/bottle.

5.5.2 Site #5, Set I-B

Site #5 Set I-B microcosms were prepared in the same manner as Set I-A, with the following key differences. With Set I-B, the bottles were purged with oxygen-free N_2 to remove residual hydrogen, which was present in the headspace of the bottles from the atmosphere of the anaerobic chamber. The bottles were purged for 10 min with high oxygen-free N_2 for two consecutive days. After nine days of incubation, 1.0 μmol/bottle (± 0.2 μmol/bottle) of neat VC gas was added to all of the microcosms, and approximately 0.2 μmol/bottle of [^{14}C]VC was added to five live microcosms and one autoclaved control. Resazurin was not added to these microcosms and they were incubated outside of the anaerobic chamber.

Microcosms in this subset were incubated for 537 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the six bottles that received [^{14}C]VC. Methane output in the live microcosms was below 1 μmol/bottle.

5.5.3 Site #5, Set I-C

Site #4 Set I-C microcosms were prepared in the same manner as the Set I-A bottles, except that a difference source of soil was used (Kings Bay KBA). Based on the change in the color of the groundwater from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. The microcosms were incubated for 539 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the six bottles that received [^{14}C]VC. Methane output started within the first 200 days of incubation and averaged 79 μmol/bottle.

5.5.4 Site #5, Set I-D

Site #5 Set I-D microcosms were prepared in the same manner as the Set I-B bottles, except that a difference source of soil was used (Kings Bay KBA). The microcosms were incubated for 542 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the six bottles that received [^{14}C]VC. Methane output in the live microcosms averaged 31 μmol/bottle, with most of the activity occurring after day 200.

5.6 SITE #6

For Site #6, only one type of soil was available, without groundwater. Similar to Site #5, all of the microcosms received 20 g of sediment + 6 mL of autoclaved DDI water. Two subsets were prepared (A and B), with one set having the headspace purged and the other set not purged. Enough sediment was available to prepare only six bottles per subset, four of which were live and two of which were autoclaved.

5.6.1 Site #6, Set I-A

Microcosms were prepared in the anaerobic chamber without amendments and without purging. After six days of incubation, 0.9 μmol/bottle (± 0.0 μmol/bottle) of neat VC gas was added to all of the microcosms, and approximately 0.2 μmol/bottle of [^{14}C]VC was added to two of the live microcosms and one of the autoclaved controls. Resazurin was added to serve as a redox indicator. Based on the change in the color of the DDI water from pink to clear shortly after all of the bottles were started, the redox level was below -110 mV for the majority of the incubation period. Microcosms in this set were incubated in the anaerobic chamber.

Microcosms in this subset were incubated in the anaerobic chamber for 667 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the three bottles that received [^{14}C]VC. Methane output in the live microcosms averaged 53 μmol/bottle.

5.6.2 Site #6, Set I-B

Site #6 Set I-B microcosms were prepared in the same manner as Set I-A, with the following key differences. With Set I-B, the bottles were purged with oxygen-free N_2 to remove residual hydrogen, which was present in the headspace of the bottles from the atmosphere of the anaerobic chamber. The bottles were purged for 10 min with high oxygen-free N_2 for two consecutive days. After six days of incubation, 0.9 μmol/bottle of neat VC gas was added to all of the microcosms, and approximately 0.2 μmol/bottle of [^{14}C]VC was added to two of the live microcosms and one autoclaved control. Resazurin was not added to these microcosms and they were incubated outside of the anaerobic chamber.

Microcosms in this subset were incubated for 667 days. There was no indication of VC biodegradation, either via reduction or oxidation (data not shown). Losses of VC from the live bottles were similar to those from the autoclaved controls. Due to the lack of any apparent VC biodegradation, no effort was made to evaluate the distribution of ^{14}C in the three bottles that received [^{14}C]VC. Methane output in the live microcosms averaged 66 μmol/bottle.

5.7 SITE #7

For Site #7, first-flush groundwater was provided from four locations; soil was not available. Six subsets of microcosms were prepared (IA, IB, IIA, IIB, IIIA, IIIB) Based on promising results from several of the microcosms, enrichments were also prepared.

5.7.1 Site #7, Set I-A

This set of microcosms was prepared with 100 mL of first flush groundwater from well E-14A. The groundwater contained low amounts of TCE and cDCE (i.e., 0.16 and 0.75 mg/L, respectively), to which VC was also added. Groundwater from this site arrived four weeks prior to microcosm construction and was stored at 4°C during this period. The COD of groundwater was below detection (10 mg/L), suggesting that there was little or no electron donor available for reductive processes. The sulfate and nitrate concentrations were 264 mg/L and 7.1 mg as N/L, respectively. The TEAs tested were oxygen, nitrate, Fe(III), Fe(III)-EDTA, Mn(IV), AQDS, and sulfate. A treatment with glucose added was also tested, replicating the approach used by Hata et al. (2003) to evaluate anaerobic oxidation of cDCE and VC.

VC was consumed without delay in the two bottles that were amended with pure oxygen. During 290 days of incubation, three to seven additions of VC (ca. 2 μmol/bottle) were consumed, suggesting that the groundwater contained microbes capable of using VC as a sole carbon and energy source. Oxygen was consumed concurrently with VC. In both bottles, cDCE was also consumed, suggesting possible aerobic cometabolic activity with VC serving as the primary substrate (Verce et al., 2002). Over 345 days of incubation, VC declined by 19% in an autoclaved control bottle, demonstrating that the substantially higher consumption rate of VC in the live bottles was a biotic process. The low initial level of cDCE in the autoclaved bottle persisted throughout the incubation.

Results for the three unamended microcosms are shown in Figure 5.7. Following 38 days of incubation in bottle #1A, VC was completely consumed with no commensurate increase in ethene or ethane (Figure 5.7a). Since the bottles were prepared under anaerobic conditions, this activity was suggestive of anaerobic oxidation of VC. Following the consumption of a second addition of VC in bottle #1A, 4.0 mL was removed to construct enrichment bottles E7-I (see below) and was replaced with 4.0 mL of groundwater (from well E-14A; Table 2.2). The third addition of VC was consumed even more rapidly, also without ethene or ethane formation. Once the fourth addition of VC was consumed by day 98, one half the volume of the microcosm was removed (50 mL) in order to start bottle #1B (Figure 5.7b); 50 mL of groundwater was added to both bottles so that the total liquid volume was 100 mL. Up to this point, 15 μmol of VC was consumed in bottle #1A.

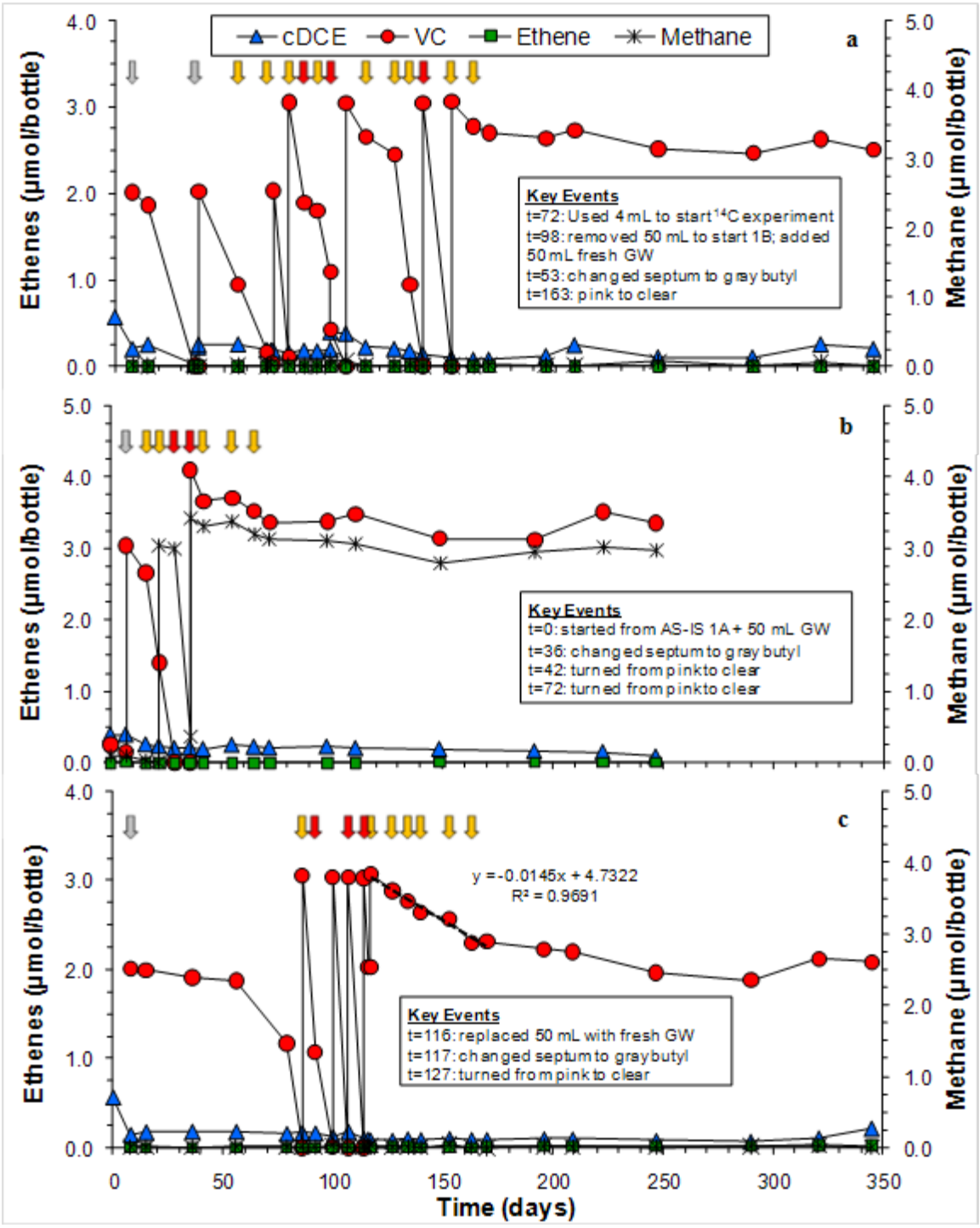


Figure 5.7. Microcosm results for Site #7, Set I-A, unamended, **a)** bottle #1A (M7-I-A-UA-1A); **b**) bottle #1B (M7-I-A-UA-1B); and **c**) bottle #2 (M7-I-A-UA-2). Arrows indicate when O_2 was measured and the results: ⇩ = below detection; ⇩ = present at 2.0-5.3 µmol/bottle; and ⇩ =present at > 5.3 µmol/bottle.

On day 98, in addition to adding VC, resazurin was added to bottle #1A. The color of the groundwater turned pink, indicating the redox level was greater than -110 mV. A fifth addition of VC was rapidly consumed and the groundwater remained pink. This suggested that leakage of oxygen into the bottle may have been responsible for consumption of the VC. Consistent with this concern, oxygen was monitored periodically between days 36 and 163 and was found at levels above detection, mainly in the range of 2.0-5.3 µmol/bottle (indicated by the yellow arrows in Figure 5.7a). The highest oxygen level measured was 13 µmol on day 98 (indicated by the red arrow in Figure 5.7a).

To address the possibility that oxygen was leaking into bottle #1A via the Teflon-faced septum, it was replaced on day 153 with a slotted grey butyl rubber septum and more VC was added. On day 163 the color of the groundwater changed from pink to clear in conjunction with the soil changing from an earthy tone to black (most likely due to formation of iron sulfides), indicating lower redox conditions had developed. Thereafter, the rate of VC consumption decreased significantly, with most of the additional consumption occurring in the first 43 days after changing the septum. The data in Figure 5.7a extends to day 345; one additional headspace sample was taken on day 957 and no additional VC was consumed (data not shown).

As in bottle #1A, the first two additions of VC (totaling 3.2 µmol/bottle) were rapidly consumed in bottle #1B, without accumulation of ethene or ethane (Figure 5.7b). On day 36, the septum was changed to slotted gray butyl rubber (corresponding to day 153 in bottle #1A). The subsequent rate of VC consumption was much slower, corresponding to the time when the resazurin turned clear. Also, oxygen was monitored periodically between days 16 and 65 and was found at levels above detection, primarily in the range of 2.0-5.3 µmol/bottle (indicated by the yellow arrows in Figure 5.7b). The highest oxygen level measured was 18 µmol on day 29 (indicated by the red arrow in Figure 5.7b). To further address the possibility that oxygen leakage was responsible for VC consumption, methane was added to bottle #1B on day 36. Since anaerobic bio-oxidation of methane is uncommon, while aerobic bio-oxidation is nearly ubiquitous, the intent of adding methane was to evaluate the potential for oxygen leakage; i.e., if methane remained constant, it would suggest that anaerobic conditions existed in the bottle. Instead, the first addition of methane was rapidly consumed, in agreement with the likelihood that oxygen was leaking in. The second addition of methane was made after changing the septum and this time the methane persisted, along with the VC. When the resazurin turned from pink to clear on day 42, the soil changed from an earthy tone to black (presumptively iron sulfides), indicating anaerobic conditions. During the time interval when the confidence level rose that anaerobic conditions existed in bottle #1B, consumption of VC slowed significantly. The data in Figure 5.7b extends to day 247; one additional headspace sample was taken on day 859 and no additional VC was consumed (data not shown).

Although activity on VC was slower to develop in bottle #2 (Figure 5.7c), the rate of consumption was quite high once it started. After 116 days of incubation, a total of 12 µmol of VC was consumed, without an increase in ethene or ethane. At that point, 50 mL was removed and sent to Dr. Stephen H. Zinder at Cornell University for further analysis. The 50 mL removed was replaced by 50 mL of groundwater (well E-14A) and the next day the septum was changed to gray butyl rubber. Oxygen was monitored periodically between days 86 and 163 and was found at levels above detection, primarily in the range of 2.0-5.3 µmol/bottle (indicated by the yellow arrows in Figure 5.7c). The highest oxygen level measured was 10 µmol on day 107.

Resazurin was added to bottle #2 on day 100; the color of the groundwater turned clear on day 127 and the soil changed from an earthy tone to black. Between days 127 and 170, when the groundwater was clear and conditions were presumptively anaerobic within bottle #2, VC decreased at a slow but statistically significant rate of 0.0145 µmol/d. Thereafter, no further VC consumption occurred, through day 345.

Unlike the oxygen amended bottles, the low level of cDCE that was initially present persisted in the presumptively anaerobic unamended bottles (Figure 5.7). Thus, if cDCE was removed via aerobic cometabolism in the aerobic treatment microcosms, a similar process was not occurring in the unamended bottles, even with consumption of methane in bottle #1-B (Figure 5.7b). Methane is a well-known primary substrate for aerobic cometabolism of cDCE (Anderson and McCarty, 1997).

Results for the duplicate microcosms amended with nitrate are shown in Figures 5.8 (bottle #2) and 5.9 (bottle #1). In bottle #2, a high rate of VC consumption started after day 210, without accumulation of ethene or ethane. When this microcosm was respiked with VC on day 248, resazurin was added and the groundwater turned pink. It remained pink throughout the remainder of the incubation period. After three more additions of VC were consumed (totaling 7.2 µmol), methane was added along with VC on day 315. Shortly after the VC was consumed, most all of the methane was consumed. This suggested the likelihood of oxygen leakage into the bottle. Oxygen was monitored periodically between days 248 and 376; three of the six measurements were above detection, in the range of 2.0-5.3 µmol/bottle; three of the measurements were below detection. The highest oxygen level measured was 22 µmol on day 248. After three additions of VC were consumed (totaling 7.2 µmol), methane was added along with VC on day 315. Shortly after the VC was consumed, most all of the methane was consumed. To reduce the chances for oxygen to diffuse into the bottle, beginning on day 355, it was kept in the anaerobic chamber at all times. VC and methane were added and headspace samples were removed while the bottle was in the chamber. The fifth addition of VC decreased more slowly, followed by a pause in the monitoring. When it was checked next on day 550, all of the VC was consumed. However, due to an oversight, more VC was not added until 664, along with a second addition of methane. Both compounds subsequently decreased, although at a slower rate than had previously been observed. On day 1002, the entire contents of the bottle were used as inoculum to develop enrichment cultures (section 5.7.7).

Beginning on day 776, the method used to measure nitrate and sulfate was modified. The Dionex column was changed from AS23 to AS9-HC. The latter gave more reliable results for standard additions to samples (data not shown). With the AS9-HC column, the concentration of nitrate in the bottle was higher, but still well below the amount added (Figure 5.8b), indicating nitrate had been consumed. From day 776 to 1002, the average concentration of nitrate was 0.45 mM, even though 0.68 mM of additional nitrate was added over this interval. There was considerably more scatter in the results for sulfate, with no clear trend for consumption. This is consistent with the fact that the groundwater remained pink during the incubation period, indicating the redox level was too high for sulfate reduction.

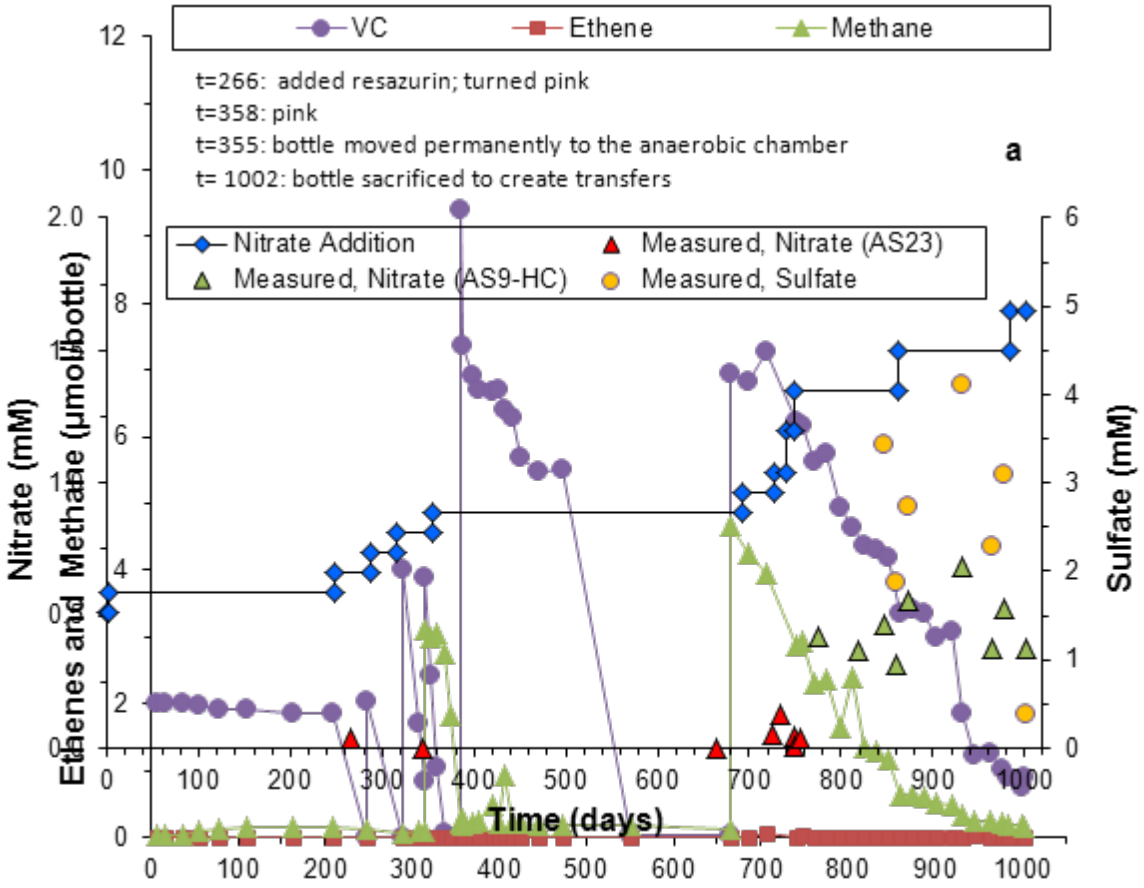
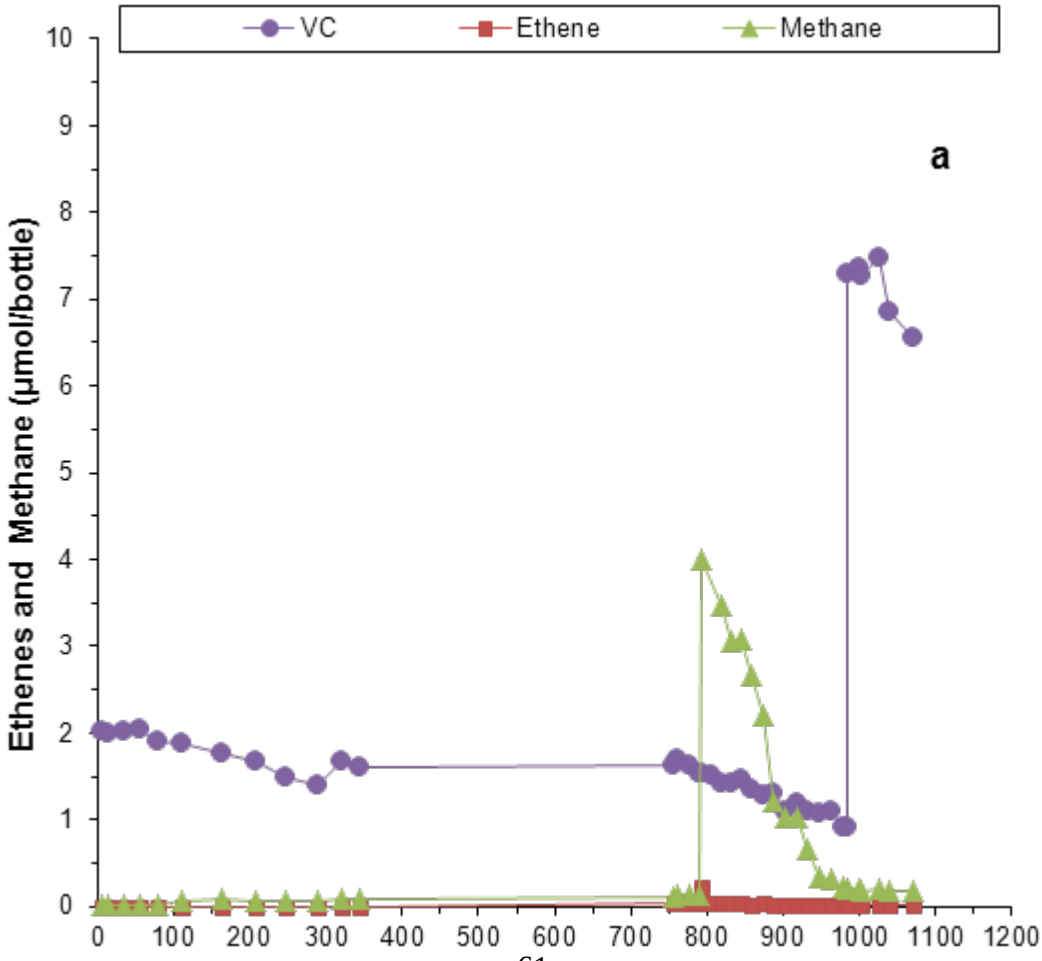


Figure 5.8. Microcosm results for Site #7, Set I-A, amended with NO_3^-, bottle #2 (M7-I-A-NO_3-2) for **a)** VOCs; and **b)** nitrate added and measured levels of nitrate and sulfate.



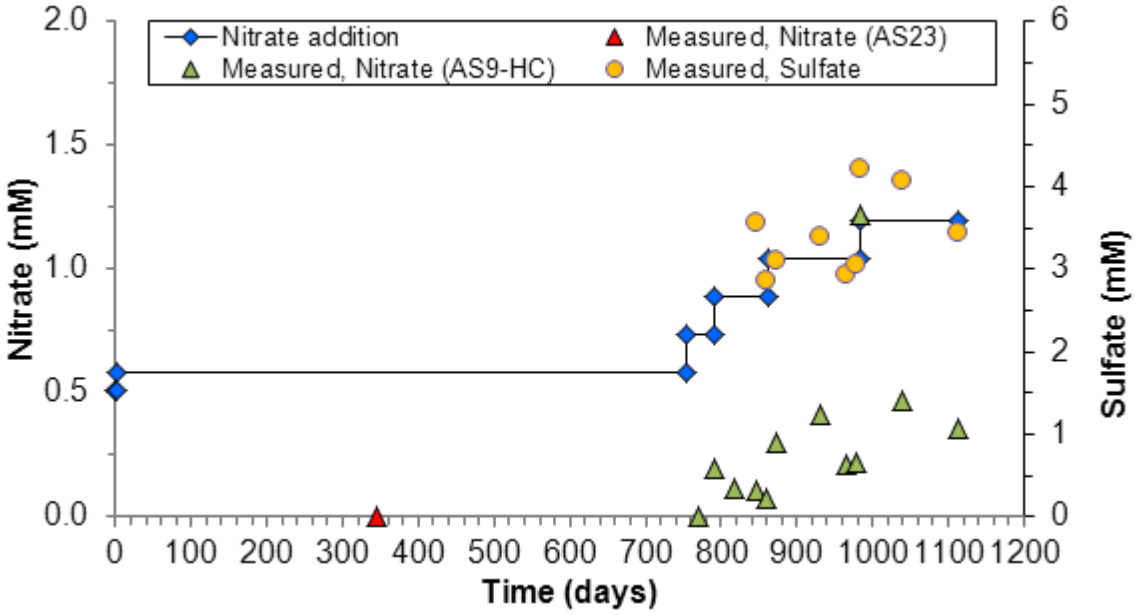


Figure 5.9. Microcosm results for Site #7, Set I-A, amended with NO_3^-, bottle #1 (M7-I-A-NO_3-1) for **a)** VOCs; and **b)** nitrate added and measured levels of nitrate and sulfate.

In nitrate-amended bottle #1, there was no appreciable decrease in VC through day 792, when methane was added. Methane decreased over the next six months at a similar rate as in bottle #1 for a similar time interval, although VC decreased at a much slower rate. A second addition of VC (without methane) on day 984 decreased at a higher rate. Since day 769, when nitrate measurements were started in bottle #1, 0.61 mM of nitrate was added, while the measured nitrate level on day 1112 was 0.36 mM, indicating that nitrate was consumed over the time period when methane and VC were consumed. The groundwater color was pink over this interval, indicating it was not conducive to sulfate reduction, as confirmed by the lack of a decreasing trend in the sulfate concentration.

Results for the two Site #7 Set I-A microcosms amended with Fe(III)-EDTA are shown in Figure 5.10. During the first 288 days of incubation in bottle #1 (Figure 5.10a), the extent of decrease in VC was no greater than in the autoclaved controls (data not shown). However, by day 321, the VC was completely depleted with no increase in ethene or ethane. When the microcosm was respiked with VC on the same day, resazurin was added; the groundwater turned pink and remained so for the remainder of the incubation period (days 321-978). Methane was also added on day 321. As mentioned above, the intent of adding methane was to serve as a biotic indicator of oxygen leakage, since anaerobic oxidation of methane is uncommon. As soon as the VC was consumed, there was a 77% decrease in methane between days 337 and 355. On the occasions when oxygen was measured (days 247-550), it was usually below detection, although levels in the range of 2.0-5.3 µmol/bottle were present several times. This microcosm was permanently moved to the anaerobic chamber from day 355 onward. Thereafter, VC decreased by 46%, and methane by 40% through day 978. Results for bottle #2 were similar (Figure 5.10b). Fe(III)-EDTA was added when the microcosms were prepared and each time they were spiked with VC; however, no attempt was made to quantify the extent of Fe(II) formation.

There was no evidence of VC biodegradation in the duplicate Fe(III) amended microcosms, over 345 days of incubation. The low amounts of cDCE initially present in the groundwater also persisted. Fe(III) was added only at the start; due to the lack of activity on VC, no further additions of Fe(III) were made. No attempt was made to quantify the amount of Fe(III) that may have been reduced to Fe(II).

Likewise, there was no evidence of VC biodegradation in the duplicate Mn(IV) amended microcosms, over 345 days of incubation. The low amounts of cDCE initially present in the groundwater also persisted. Mn(IV) was added only at the start; due to the lack of activity on VC, no further additions of Mn(IV) were made. No attempt was made to quantify the amount of Mn(IV) that may have been reduced to Mn(II).

The duplicate microcosms amended with AQDS behaved very differently. In one, there was no significant loss of VC during 345 days of incubation. In the other microcosm, the initial dose of VC was completely consumed between days 209 and 250, without an increase in ethene or ethane (Figure 5.11). The next two additions of VC were also consumed. Methane was added along with the fourth addition of VC. In this instance, consumption of methane occurred concurrently with consumption of the VC. Following the fifth addition of VC and second addition of methane, both compounds initially decreased at a rate similar to the previous additions but then the rate slowed considerably for VC and consumption of methane stopped. In the interval between days 308 and 376, oxygen was measured six times; on two occasions,

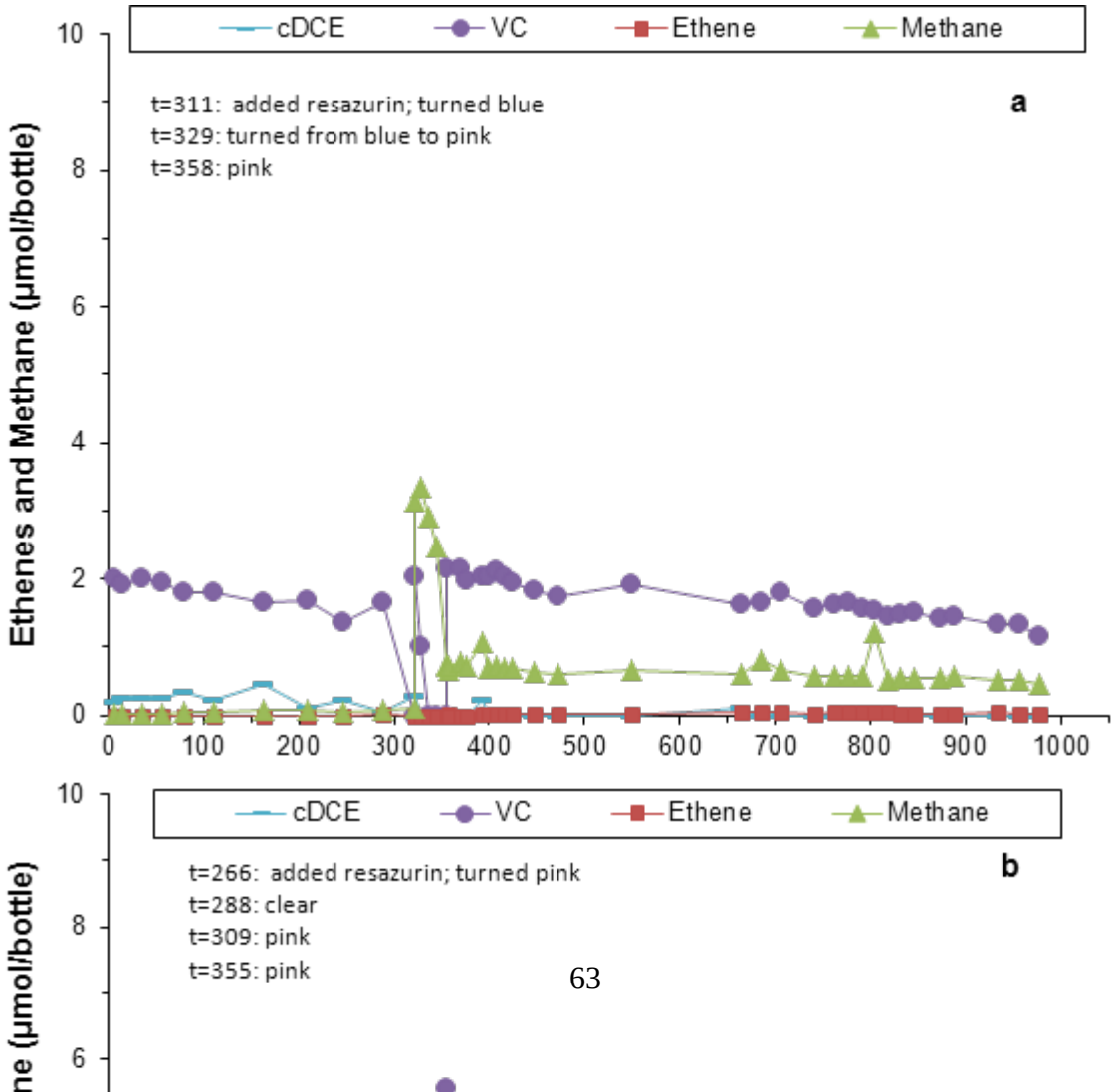


Figure 5.10. Microcosm results for Site #7, Set I-A, amended with Fe(III)-EDTA for bottle **a)** #1 (M7-IA-Fe(III)-EDTA-1) and **b)** #2 (M7-IA-Fe(III)-EDTA-2).

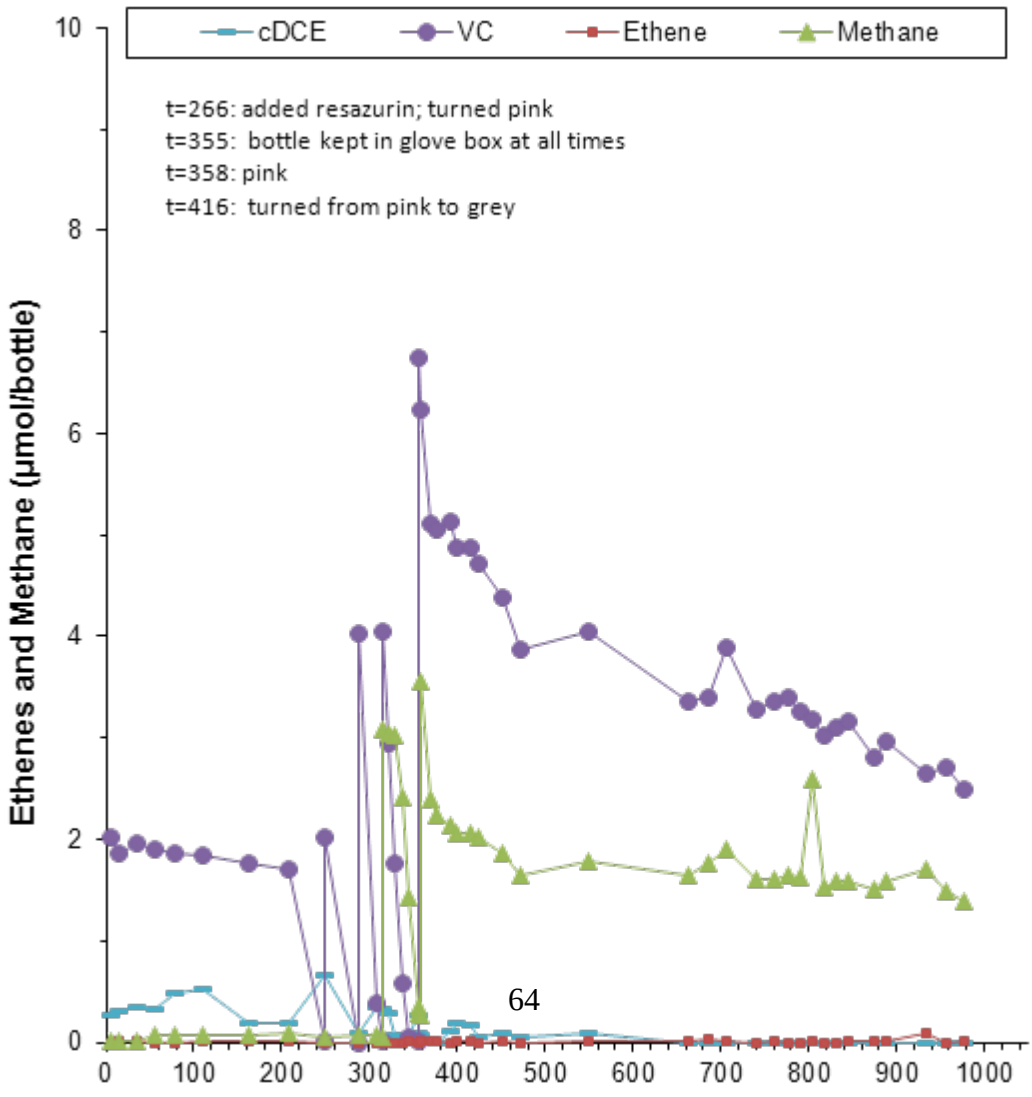


Figure 5.11. Microcosm results for Site #7, Set I-A, amended with AQDS for bottle #2 (M7-IA-AQDS-2).

oxygen was below detection and at the other times oxygen was detected at 2.0-5.3 µmol/bottle. This microcosm was moved permanently inside the anaerobic chamber from day 355 onward, corresponding to the decreased activity on VC and methane. AQDS was added each time VC was added; however, no attempt was made to determine if the AQDS was being reduced.

There was no evidence of VC biodegradation in the duplicate sulfate-amended microcosms, over 345 days of incubation. The low amounts of cDCE initially present in the groundwater also persisted. Sulfate was present in the groundwater and was also added at time zero. Due to the lack of activity on VC, no further additions of sulfate were made and no attempt was made to quantify the amount of sulfate consumed.

Likewise, there was no evidence of VC biodegradation in the duplicate microcosms to which glucose was added, over 345 days of incubation. The low amounts of cDCE initially present in the groundwater also persisted. There was no accumulation of methane, which was somewhat surprising given the high initial dose of glucose (10 g/L).

In summary, VC consumption without ethene or ethane accumulation occurred in seven of the 17 Site #7, Set I-A (presumptively) anaerobic microcosms: three unamended (Figure 5.7) two nitrate-amended (Figures 5.8 and 5.9); one Fe(III)-EDTA amended (Figure 5.10), and one AQDS-amended (Figure 5.11). However, several lines of evidence suggest that the consumption of VC in these bottles may have been due to oxygen leakage and hence aerobic oxidation, rather than anaerobic oxidation. First, when VC consumption started in these bottles, oxygen was usually detected. Occasionally oxygen was present at levels high enough to be quantified (i.e., above 2.0 µmol/bottle), but most often it was detected at levels that were below quantification. Second, whenever methane was added as a biotic tracer along with VC, it too was consumed; it is more likely for methane to be oxidized aerobically than anaerobically. Third, during periods when VC was rapidly consumed and resazurin was present, the groundwater was pink, indicating a redox level above -110 mV. Fourth, whenever the septa were changed from Teflon-faced red rubber to slotted gray butyl rubber, the rate of VC consumption either slowed considerably or stopped, suggesting that the grey butyl rubber is less permeable to oxygen leakage. Fifth, when the bottles were moved permanently to the anaerobic chamber, the rate of VC and methane oxidation slowed or stopped. And sixth, the Zinder lab was unable to replicate any anaerobic VC biodegradation using the contents from one of the positive unamended microcosms. Another feature of the biotic activity on VC that was unexpected was the timing; in the four active bottles

that were amended with a terminal electron acceptor, biodegradation of VC started on day 209, making the onset of activity surprisingly uniform. However, there were no changes in how these bottles were maintained that might explain this.

There are several behaviors in the positive microcosms that seem to conflict with oxygen leakage as the explanation for VC biodegradation. Since all of the microcosms were treated the same, one would expect all of the bottles to be subject to similar levels of leakage and therefore similar levels of VC biodegradation; that did not occur. Only seven of the 19 microcosms tested exhibited VC biodegradation. Also, the onset of VC biodegradation was consistently abrupt and then ended abruptly. One might expect that if oxygen leaked in at a slow but consistent rate, the rate of leakage would control the rate of VC biodegradation, as demonstrated by Gossett (2010) using permeation tubes to deliver oxygen. Regardless, the burden of proof requires unequivocal demonstration that oxygen was not responsible for VC biodegradation; these results do not satisfy that requirement.

5.7.2 Site #7, Set I-B

This set of microcosms was prepared with 100 mL of first flush groundwater from Site #7, well TW-4A. The groundwater contained trace amounts of TCE and low amounts of cDCE (i.e., 0.11 and 0.33 mg/L, respectively), to which VC was also added. Groundwater from this site arrived four weeks prior to microcosm construction and was stored at 4°C during this period. The same treatments described above for Set I-A were prepared for Set I-B (also in duplicate, except for a single autoclaved control). The COD of groundwater from well TW-4A was 103 mg/L, suggesting that there was donor available for reductive processes. The sulfate and nitrate concentrations were 95 mg/L and 6.7 mg as N/L, respectively. Acetate in the groundwater was 11 mg/L, which was most likely due to the incomplete oxidation of sodium lactate that was injected at Site #7 to drive reductive dechlorination.

As with Set I-A, VC was consumed without delay in the two bottles that were amended with oxygen. During 286 days of incubation, eight to nine additions of VC (ca. 2 µmol/bottle) were consumed, suggesting that the groundwater contained microbes capable of using VC as a sole carbon and energy source. A large decrease in oxygen occurred initially in both bottles which was most likely due to the COD in the groundwater. Thereafter, the stoichiometry of aerobic VC oxidation was 1.7 µmol of oxygen per µmol of VC (i.e., for days 92-286); this compares to 2.5 µmol of oxygen per µmol of VC without accounting for cell synthesis. Over 350 days of incubation, VC declined by 34% in an autoclaved control bottle, demonstrating that the substantially higher consumption rate of VC in the live bottles was a biotic process.

In the anaerobic treatments with TEAs added, VC biodegradation occurred in nearly all of the bottles via stoichiometric reductive dechlorination to ethene; there was no indication of anaerobic bio-oxidation of VC. There was variability among treatments in the rate and extent of VC reduction, perhaps due to the extent of competing terminal electron acceptor usage. Nevertheless, reductive dechlorination was the only type of VC biodegradation observed. Methane output was highest in the unamended treatment (110 µmol/bottle versus less than 10 in the TEA amended treatments).

In the treatment amended with a high concentration of glucose (10 g/L), VC did not undergo biodegradation. Although methane output was relatively low (i.e., below 10 µmol/bottle, relative to 16,700 µmol possible if all the glucose was converted), gas production was so significant (presumptively hydrogen and CO_2) that the headspace pressure became excessive (to the extent that the septa began to bulge), necessitating that both bottles be vented on day 37; bottle #2 also needed to be vented again on day 57.

5.7.3 Site #7, Set II-A

This set of microcosms was prepared with 100 mL of first flush groundwater from well IP-05 (Table 2.2). The same treatments described above for Set I-A were prepared for Set II-A (also in duplicate, except for a single autoclaved control). The groundwater contained trace amounts of TCE and cDCE (i.e., 0.07 and 0.65 mg/L, respectively), to which VC was also added. Groundwater from this site arrived four weeks prior to microcosm construction and was stored at 4°C during this period. The COD of groundwater from well IP-05 was 132 mg/L, suggesting that there was donor available for reductive processes.

As with Set I-A, VC was consumed without delay in the two bottles that were amended with oxygen. During 253 days of incubation, five additions of VC (ca. 2 µmol/bottle) were consumed, suggesting that the groundwater contained microbes capable of using VC as a sole carbon and energy source. Oxygen consumption was highest over the first 144 days, suggesting that COD in the groundwater was being consumed as well as the VC. These results demonstrated that the IP-05 groundwater contains microbes capable of aerobically oxidizing VC.

Over the same time interval, VC declined by 34% in an autoclaved control bottle, demonstrating that the substantially higher consumption rate of VC in the live bottles was a biotic process.

In the anaerobic treatments with TEAs added, there was no indication of VC biodegradation, either via reduction or oxidation. The low level of cDCE initially present also persisted and there was no methane production, in spite of a relatively high COD level in the groundwater. There was also no evidence of VC biodegradation in the treatment with a high concentration of glucose added. A modest level of methane was produced (ca. 160 µmol/bottle, which is far less than what could have been produced if all of the glucose was converted).

5.7.4 Site #7, Set II-B

This set of microcosms was prepared with 100 mL of first flush groundwater from Site #7, well IP-05 (Table 2.2). The groundwater contained trace amounts of TCE and low amounts of cDCE (i.e., 0.011 and 0.18 mg/L, respectively), to which VC was also added. Groundwater from this site arrived four weeks prior to microcosm construction and was stored at 4°C during this period. The COD of groundwater from well IP-07 was 127 mg/L, suggesting that there was donor available for reductive processes.

As with Sets I-A, I-B. and II-A, VC was consumed without delay in the two Set II-B bottles that were amended with oxygen. During 253 days of incubation, four additions of VC (ca. 2 µmol/bottle) were consumed. Oxygen consumption was highest over the first 146 days, suggesting that COD in the groundwater was being consumed as well as the VC. These results demonstrated that the IP-07 groundwater contains microbes capable of aerobically oxidizing VC.

Over the same time interval, VC declined by 48% in an autoclaved control bottle, demonstrating that the substantially higher consumption rate of VC in the live bottles was a biotic process.

In the anaerobic treatments with TEAs added, there was no indication of VC biodegradation, either via reduction or oxidation. The low level of cDCE initially present also persisted and there was negligible methane production (i.e., ,4 µmol/bottle), in spite of a relatively high COD level in the groundwater. There was also no evidence of VC biodegradation in the treatment with a high concentration of glucose added. A modest level of methane was produced in one of the bottles (ca. 600 µmol, which is far less than what could have been produced if all of the glucose was converted).

5.7.5 Site #7, Set III-A

This set of microcosms (M7-III-A) was prepared with 100 mL of first flush groundwater from Site #7, well E-14A (Table 2.2). This was the same well used to prepare the Site #7, Set I-A microcosms, although the groundwater sample was taken at a later date. An additional sample was requested based on the initially promising results with the anaerobic unamended microcosms (Figure 5.7). The groundwater contained trace amounts of TCE and low amounts of cDCE (i.e., 0.11 and 0.56 mg/L, respectively), to which VC was also added. Groundwater from this site arrived eight weeks prior to microcosm construction and was stored at 4°C during this period. The COD of groundwater from this sample of well E-14A was below the detection limit of 10 mg/L, suggesting there was little or no donor available for reductive processes.

The only treatment prepared for the Set III-A groundwater was unamended anaerobic microcosms, consisting of four bottles. Unlike the previous unamended bottles, there was no evidence of VC biodegradation in this set, either by oxidation or reduction during the 196 days of incubation. Methanogenesis did not occur.

5.7.6 Site #7, Set III-B

This set of microcosms was prepared with the same groundwater (well E-14A) as Set III-A. However, they were designated with a different set number because a different approach was used for their preparation. With the Set III-B bottles, purging was performed with nitrogen gas by inserting a cannula into the groundwater; by contrast, with the all of the other Site #7 microcosms, only the headspace was purged. One concern with the behavior of the Set I-A unamended microcosms (Figure 5.7) was the possibility that VC oxidation was due to dissolved oxygen in the groundwater, which may not have been completely removed during preparation in the anaerobic chamber. To address this concern, the groundwater in the two Set III-B bottles was directly purged, with the intent of stripping away any remaining oxygen. cDCE and TCE were removed by this more aggressive method of stripping. Nevertheless, these bottles behaved the same as Set III-A, i.e., there was no evidence of VC during the 196 days of incubation.

5.7.7 Enrichments from Site #7, Set I-A Unamended Microcosms

Based on the initially promising results for anaerobic oxidation of VC in bottle #1A of the unamended microcosms for Site #7, Set I-A (M7-I-A-UA-1A; Figure 5.7a), aliquots from this microcosm were used to prepare four enrichments (E7-I) (Table 2.2). Each bottle (70 mL total volume) received 1 mL of well-mixed liquid from bottle #1A (following 72 days of incubation)

plus 19 mL of groundwater from well E14-A. Smaller volume bottles were used (i.e., rather than 160 mL) because not enough groundwater was available to prepare larger volumes. Along with the four live bottles, two water controls were prepared. All received an initial VC dose of approximately 2.0 µmol/bottle. Two of the live bottles (#3 and #4) and the two water controls also received [^{14}C]VC (which was separated from toluene in the stock solution using the chromatographic method described in section 2.4.1).

Unlike the parent microcosm, there was no significant biodegradation of VC in the transfer bottles. Over 677 days of incubation, VC decreased by ca. 40% in the bottles with [^{14}C]VC added. A similar extent of loss occurred in duplicate water controls and in duplicate live bottles that received only unlabeled VC. On day 677, the microcosms were sacrificed for ^{14}C analysis. Less than 2% of the [^{14}C]VC added was recovered as $^{14}CO_2$ or [^{14}C]NSR (data not shown), indicating there was no anaerobic oxidation of VC in this treatment, consistent with the GC headspace results.

5.7.8 Enrichments from Site #7, Set I-A Nitrate-Amended Microcosms

Based on the promising results for anaerobic oxidation of VC in the nitrate-amended Site #7, Set I-A microcosms (section 5.7.1), especially bottle M7-I-A-NO3-2 (Figure 5.8), enrichments were prepared using samples from this bottle. The 120 mL bottles used for this transfer received 10 mL from bottle M7-I-A-NO3-2, 30 mL of groundwater from well E-14A (the same groundwater used to prepare the Site #7, Set IA microcosms), and 35 mL of mineral salts medium prepared according to Ettwig et al. (2010). Four treatments were prepared: 1) with nitrate added; 2) with nitrite added; 3) with nitrate + nitrite added; and 4) with no nitrate or nitrite added. The bottles were capped with slotted gray butyl rubber septa, removed from the anaerobic chamber, purged (10 min) with high purity N_2 (passed through a buffered solution of 20% titanium (III) chloride to remove trace levels of oxygen), and resealed. VC was added to all of the bottles and stock solutions were used to deliver 2 mM of nitrate and nitrite (150 µmol/bottle) to the appropriate treatments. This concentration was used to ensure an excess of electron acceptor with respect to the amount of VC added (8 µmol/bottle). The bottles were returned to the anaerobic chamber and remained there at all times, including during headspace sampling. On day 5 of the incubation, the pH was found to be ca. 8.6; it was decreased to ca. 7 by addition of 5 N HCl.

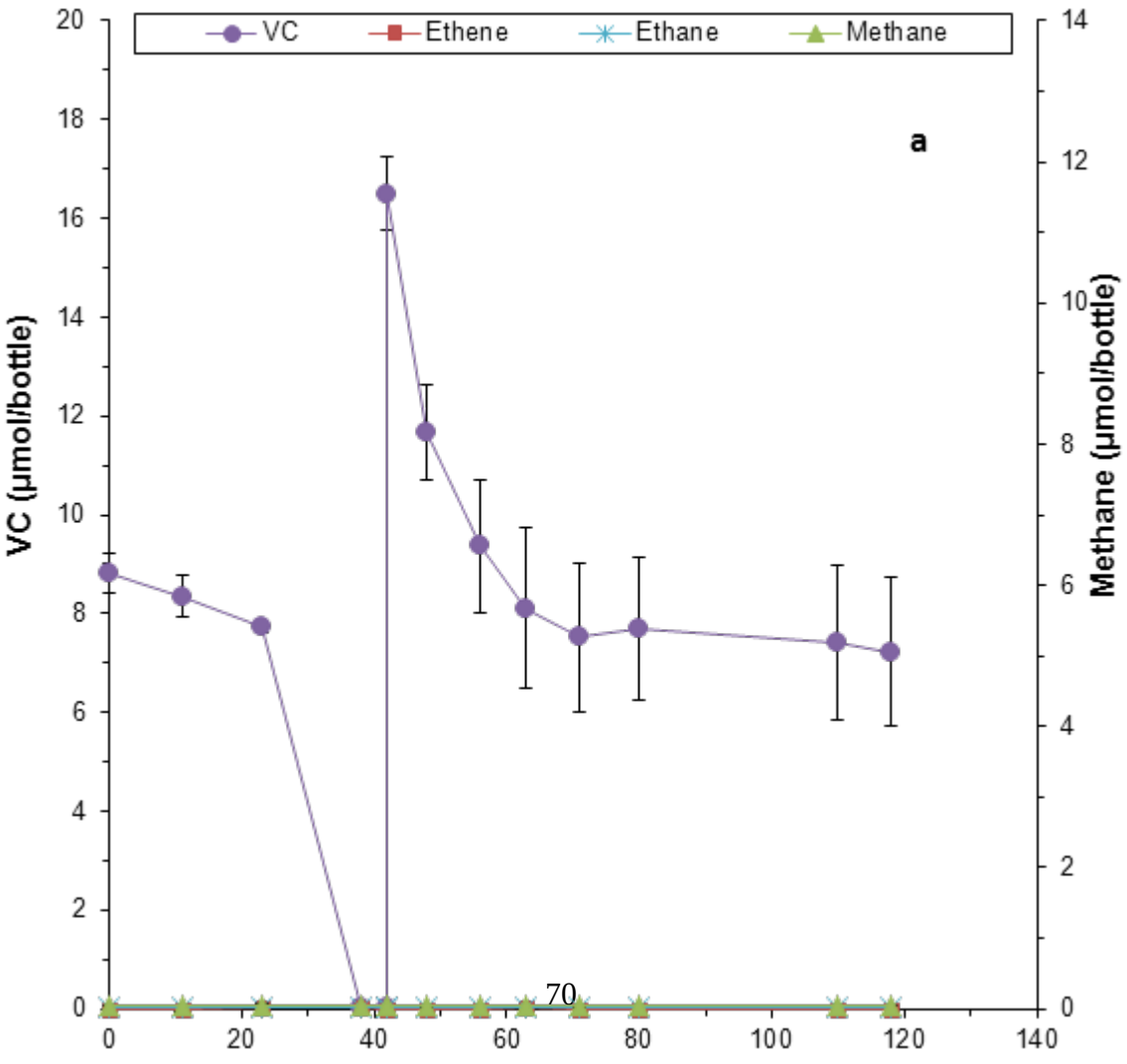
Average results for the triplicate bottles that were amended with nitrate are shown in Figure 5.12. After a lag of ca. 25 days, VC decreased to non-detect by day 42 and a second dose was added. It initially decreased at a similar rate and then degradation abruptly slowed. Approximately 18 µmol of VC was consumed, without a commensurate increase in ethene or ethane. Methanogenesis was also inhibited through the incubation period. The color of the liquid in these bottles (based on resazurin), as well as all of the others, changed from lavender to pink sometime between days 11 and 23, and remained pink thereafter. Nitrate decreased by 0.14 mM and nitrite increased by 0.08 mM, suggesting that a modest level of denitrification occurred. There was no significant change in sulfate. Headspace samples analyzed on day 376 indicated no significant further decrease in VC (data not shown).

Average results for the triplicate bottles that were amended with nitrite are shown in Figure 5.13. There was no appreciable decrease in VC over the 118 day incubation period. However, between days 23 and 110, nitrite decreased by 1.0 mM and nitrate increased by 1.0 mM; there

was no significant change in sulfate. Oxidation of nitrite to nitrate suggests that oxygen was available, in spite of the fact that the bottles were prepared in the anaerobic chamber, the headspace was purged with N_2, and the bottles were incubated at all times (including sampling) in the anaerobic chamber. Headspace samples analyzed on day 376 indicated no significant decrease in VC (data not shown).

Average results for the triplicate bottles that were amended with nitrate and nitrite are shown in Figure 5.14. The results were similar to the treatment with only nitrite added; there was no appreciable decrease in VC. However, between days 23 and 110, nitrite decreased by 1.2 mM and nitrate increased by 0.79 mM; there was no significant change in sulfate. The results confirm that nitrite was oxidized to nitrate, in spite of the steps taken to exclude oxygen from the bottles. Headspace samples analyzed on day 376 indicated no significant further decrease in VC (data not shown).

Average results for the triplicate bottles that were unamended (i.e., no nitrate or nitrite was added) are shown in Figure 5.15. Furthermore, the groundwater added to the bottles did not have any detectable nitrite and only 0.023 mM nitrate. The bottles behaved similarly to the treatment with nitrate added (Figure 5.12). Approximately 15.1 µmol/bottle of VC was consumed before degradation activity ceased. There was no increase in ethene, ethane, or methane, and no significant decrease in sulfate.



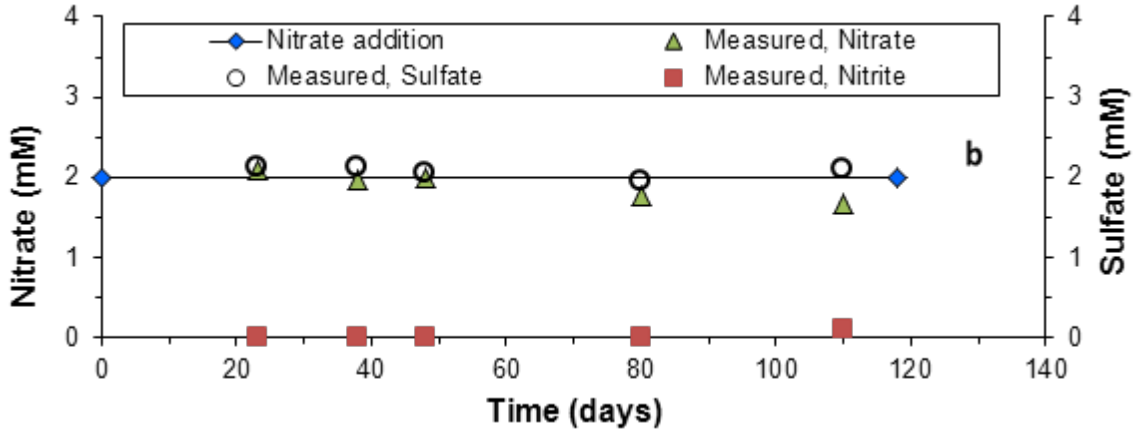
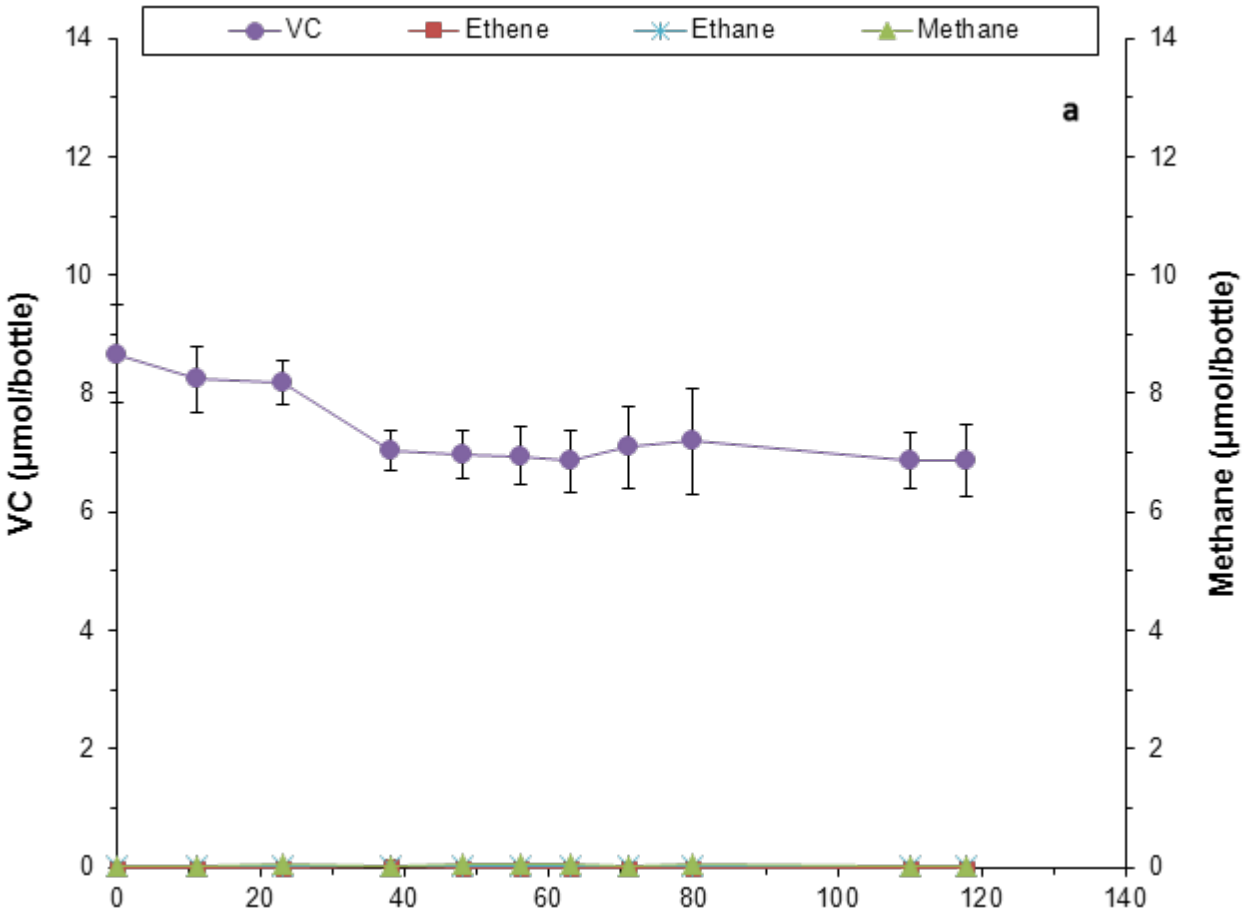


Figure 5.12. Enrichment culture derived from the first transfer of microcosm M7-I-A-NO3-2, amended with nitrate, average of triplicate bottles for **a)** VC; and **b)** nitrate, nitrite and sulfate. Error bars represent one standard deviation.



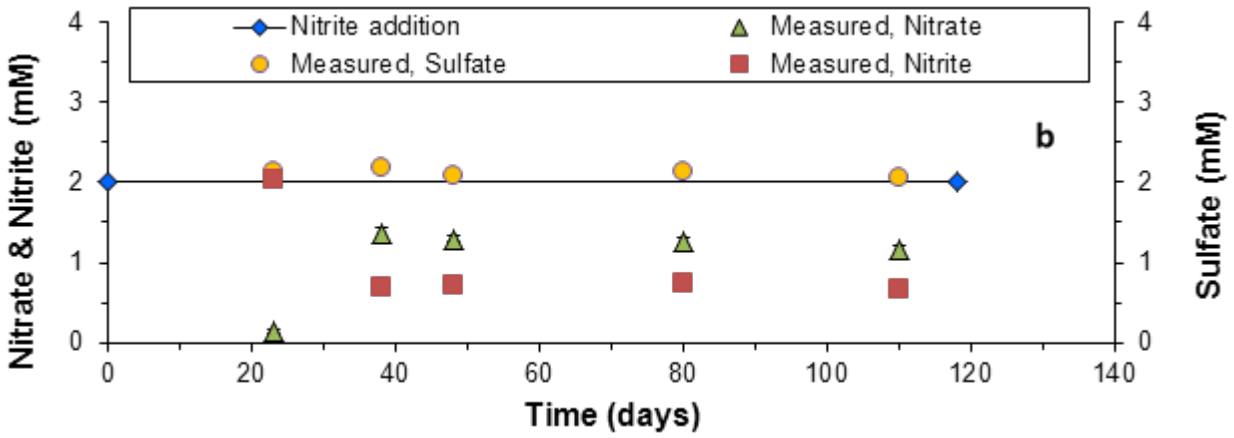
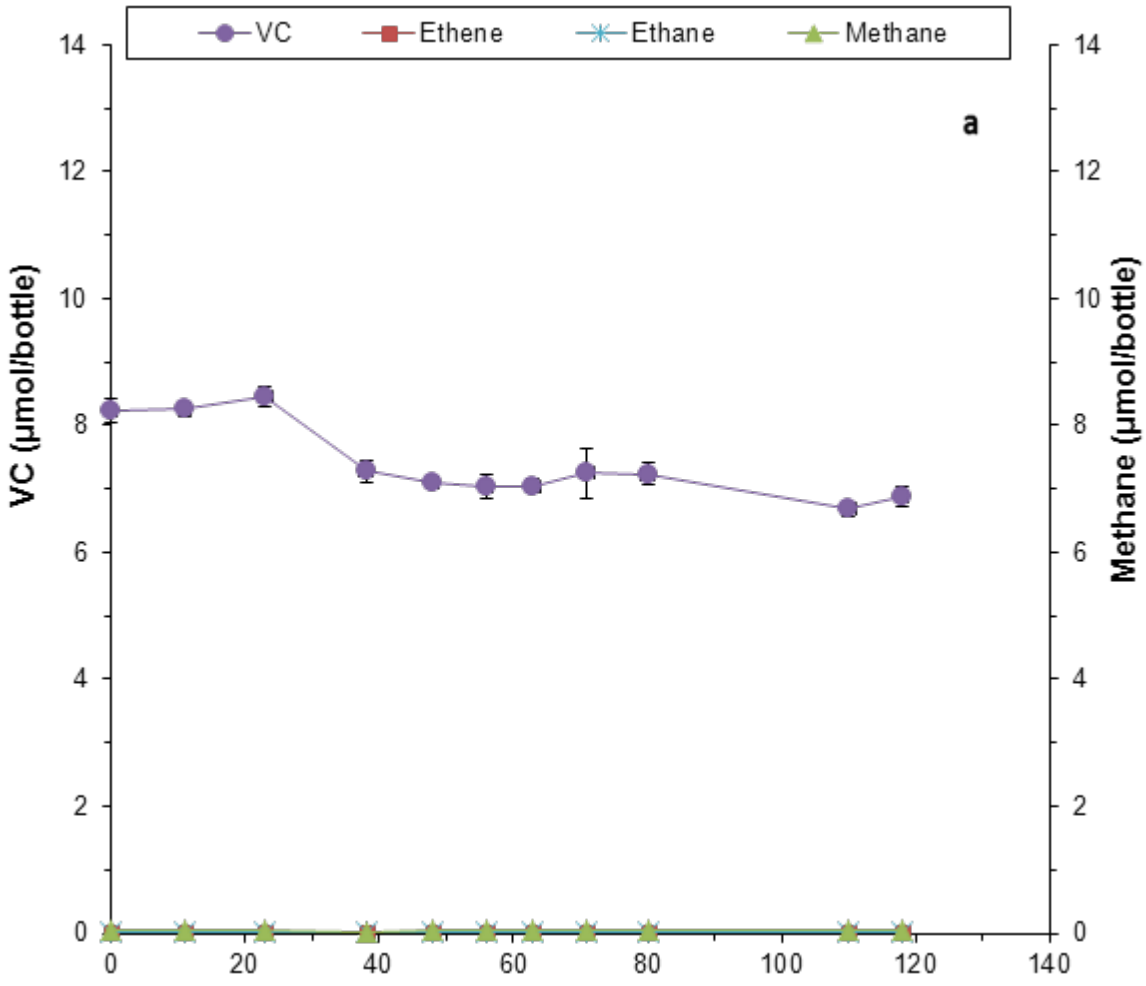


Figure 5.13. Enrichment culture derived from the first transfer of microcosm M7-I-A-NO3-2, amended with nitrite, average of triplicate bottles for **a)** VC; and **b)** nitrate, nitrite and sulfate. Error bars represent one standard deviation.



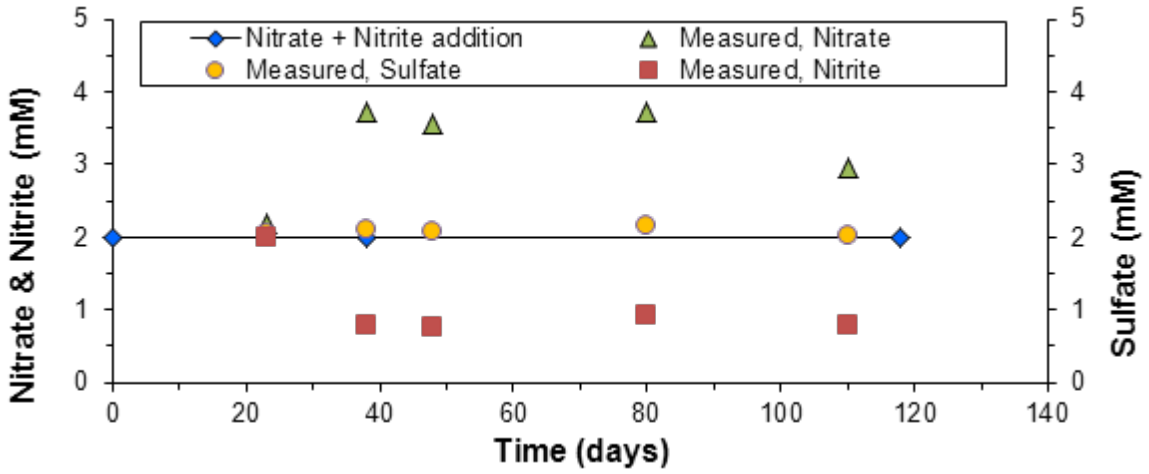
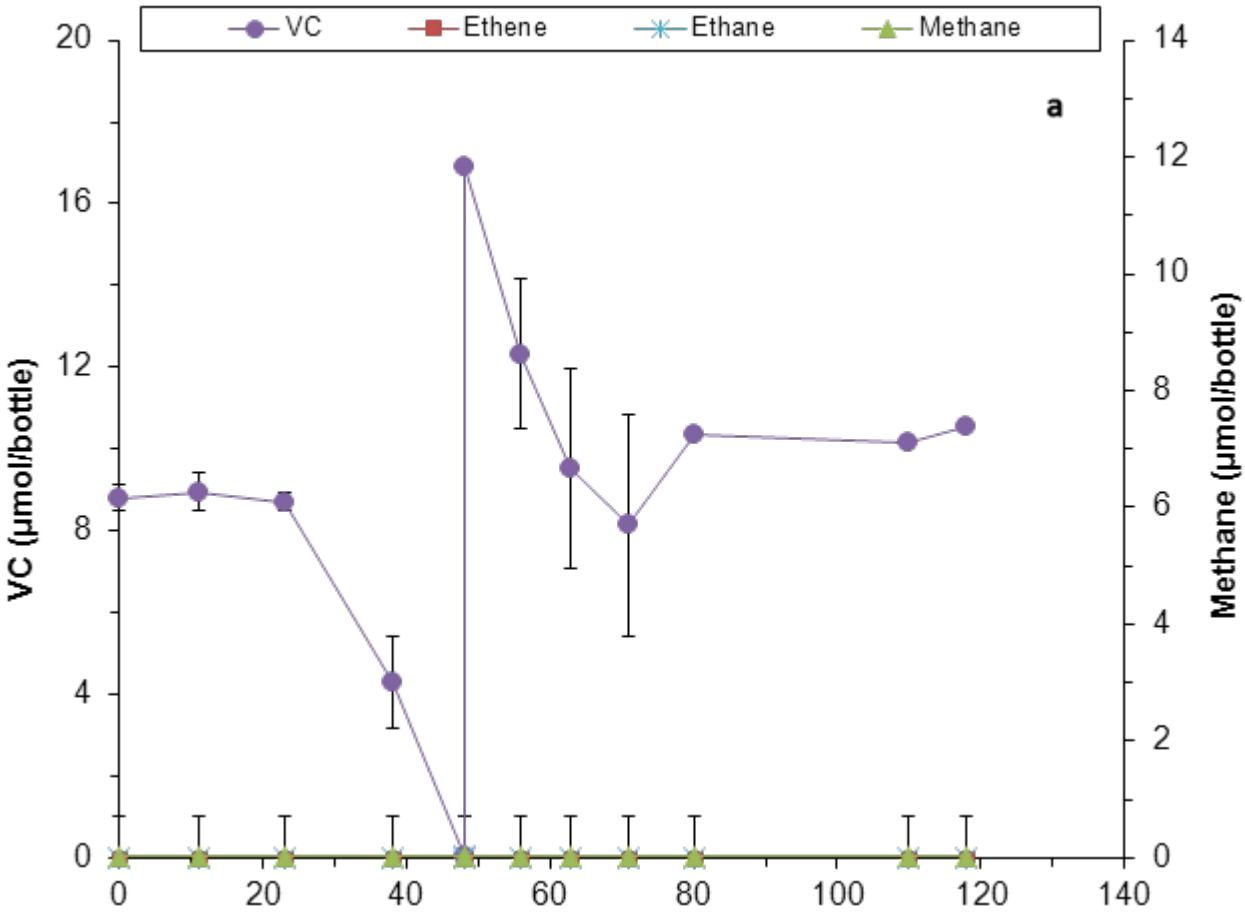


Figure 5.14. Enrichment culture derived from the first transfer of microcosm M7-I-A-NO3-2, amended with nitrate and nitrite, average of triplicate bottles for **a)** VC; and **b)** nitrate, nitrite and sulfate. Error bars represent one standard deviation.



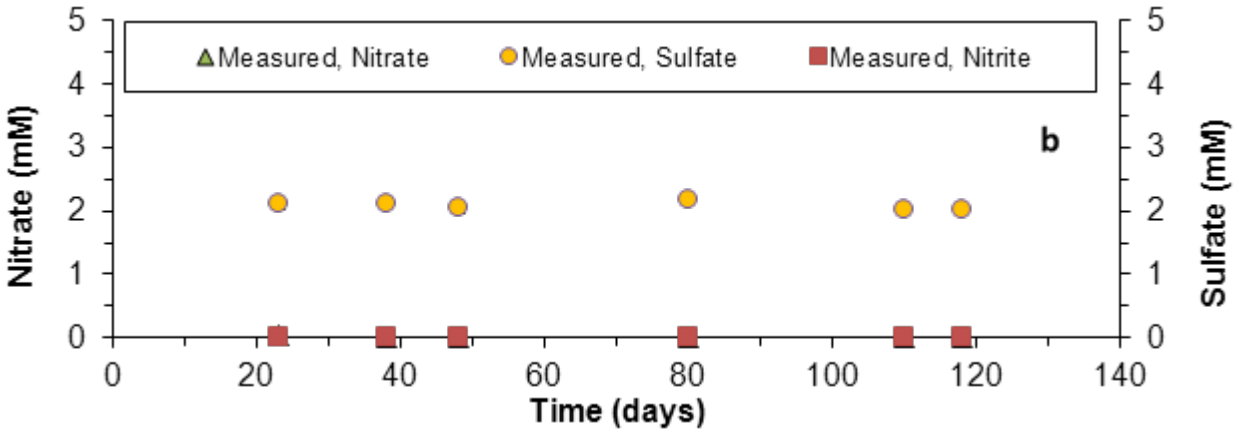


Figure 5.15. Enrichment culture derived from the first transfer of microcosm M7-I-A-NO3-2, unamended, average of triplicate bottles for **a)** VC; and **b)** nitrate, nitrite and sulfate. Error bars represent one standard deviation.

Based on apparent anaerobic oxidation of VC in the first set of transfers, a second set was prepared, following the same experimental design. Two of the bottles from the first transfer that received nitrate were used as the inoculum. The same quantities of liquid were added, i.e., 10 mL of inoculum, 35 mL of the medium described by Ettwig et al. (2010), and 30 mL of the groundwater from well E14A. However, several changes were made in the method of preparation. First, the pH of the medium was adjusted before addition to the serum bottles by purging with 5% CO_2/ 95% N_2. Second, after the bottles were prepared in the anaerobic chamber, they were sealed with the slotted grey butyl rubber septa and never removed (including for sampling). Therefore, the headspace of the bottles contained the same composition of headspace in the chamber, which at the time was ca. 4% H_2 and 96% N_2.

After 302 days of incubation, there was no appreciable decrease in VC in any of the second transfer bottles (data not shown). In the treatment without nitrate or nitrite added, the color of the liquid turned clear within three days, indicating that the presence of hydrogen in the headspace led to the development of reducing conditions. In the treatment with nitrate added, 2.1 mM of nitrate was consumed by day 44 and nitrite increased by 0.63 mM, indicating that denitrification occurred and was proceeding beyond nitrite. In the treatment with nitrite added, 2.0 mM of nitrite was consumed by day 22; no nitrate was detected. This is consistent with lower redox conditions in the bottles, with nitrite likely reduced to NO, N_2O, and/or N_2. In the treatment with nitrite and nitrate added, all of the added nitrate (2.0 mM) was consumed by day 22; nitrite increased by 1.5 mM and then started to decrease, suggesting that denitrification was proceeding beyond nitrite. In all of the treatments with nitrate and/or nitrite present, the color of the liquid remained pink.

In summary, consumption of VC in two of the treatments for the first transfer (unamended and nitrate amended) occurred without accumulation of ethene or ethane, suggesting anaerobic

oxidation. However, the fact that nitrite was oxidized to nitrate in the nitrite-amended treatment casts doubt on the presumption that the bottles were free of oxygen. Furthermore, if anaerobic bio-oxidation of VC did occur, it is unclear what the electron acceptor was, since the unamended treatment had very little nitrate and no nitrite and there was no significant decrease in sulfate. Additional precautions were taken with the second transfer cultures to further reduce the potential for oxygen contamination; however, through 302 days of incubation, no biodegradation of VC occurred. Decreases in nitrate and nitrite suggest these bottles have active denitrification, driven by hydrogen from the anaerobic chamber.

5.8 SITE #8

The project scope was expanded to include an evaluation of anaerobic oxidation of ethene. This objective was addressed with soil samples from three sites (#8, #9, and #10). The sample from Site #8 consisted of uncontaminated soil from a flower bed adjacent to the L. G. Rich Environmental Research Laboratory at Clemson University. Two sets of microcosms were prepared with this soil plus various types of media. The first set was prepared without [^{14}C]ethene, the second set was prepared with [^{14}C]ethene. Treatments included amendments with oxygen, nitrate, Fe(III), Fe(III)-EDTA, sulfate, glucose, and GLF. Enrichments were prepared with samples from the Set I microcosms.

5.8.1 Site #8, Set I

This set of microcosms was prepared with 20 g of soil and the media outlined in Table 2.1, based on the desired treatment. Soil was collected 15 to 30 cm below the surface and an effort was made to exclude organic material like leaves and roots. The soil had no record of prior contamination with chlorinated organic compounds.

During 77 days of incubation, two additions of ethene were consumed in the oxygen-amended bottles. These results confirmed that the soil contained microbes capable of oxidizing ethene under aerobic conditions. This compares to a decrease of only 11% ethene in the autoclaved controls over 510 days of incubation (data not shown).

There was no significant decrease in ethene in the nitrate-amended treatment over 230 days of incubation (data not shown). However, in the three microcosms amended with Fe(III), there was close to stoichiometric reduction of the first addition of ethene to ethane (Figure 5.16; Table 5.1). The medium contained 2700 mg/L of sodium acetate and 50 mg/L of yeast extract, so there was a considerable excess of electron donor (2991 mg/L COD) in comparison to the amount needed to reduce ethene to ethane. Concurrent with the onset of ethene reduction was a substantial output of methane (1570 µmol/bottle on day 84). In order to relieve the gas pressure in the headspace, the bottles were vented to the atmosphere on day 84. Methanogenesis slowed thereafter, so that additional venting was not needed for the duration of the incubation. Reduction of the ethene to ethane continued with the second and third additions (days 84 and 128). With the fourth addition of ethene (day 154), the amount was increased to approximately 1% of the headspace volume (40 µmol/bottle), with the intent of inhibiting reduction to ethane. Between days 161 and 230, ethene decreased at a rate of 0.0456-0.0185 µmol/bottle/d, as shown

by the trend lines in Figure 5.16. Only 38% of the decrease in ethene was attributable to an increase in ethane (Table 5.1), suggesting that the balance of ethene consumption was a consequence of some other process, such as oxidation. Over this same interval, there was no statistically significant increase in methane (based on a Student's *t*-test for the slope of the regression line for methane data, α=0.05), indicating that the decreases in ethene were not likely due to diffusive losses. Fe(III) was added when ethene was added, totaling 160 µeeq/bottle (equivalent to 13 mg COD/L). No attempt was made to quantify the amount of Fe(III) that may have been reduced to Fe(II).

The rates of ethene decrease shown in Figure 5.16 were converted to a net rate of 0.059±0.027 µM/d (Figure 5.17). The net rate was calculated by subtracting the rate of ethane formation (based on linear regression of the ethane data over the same time interval) and then converting µmol/bottle/d to µM/d using equation 2.1. The rates should be viewed with caution, however, since no evidence was obtained for conversion of ethene to products other than ethane (i.e., [^{14}C]ethene was not used).

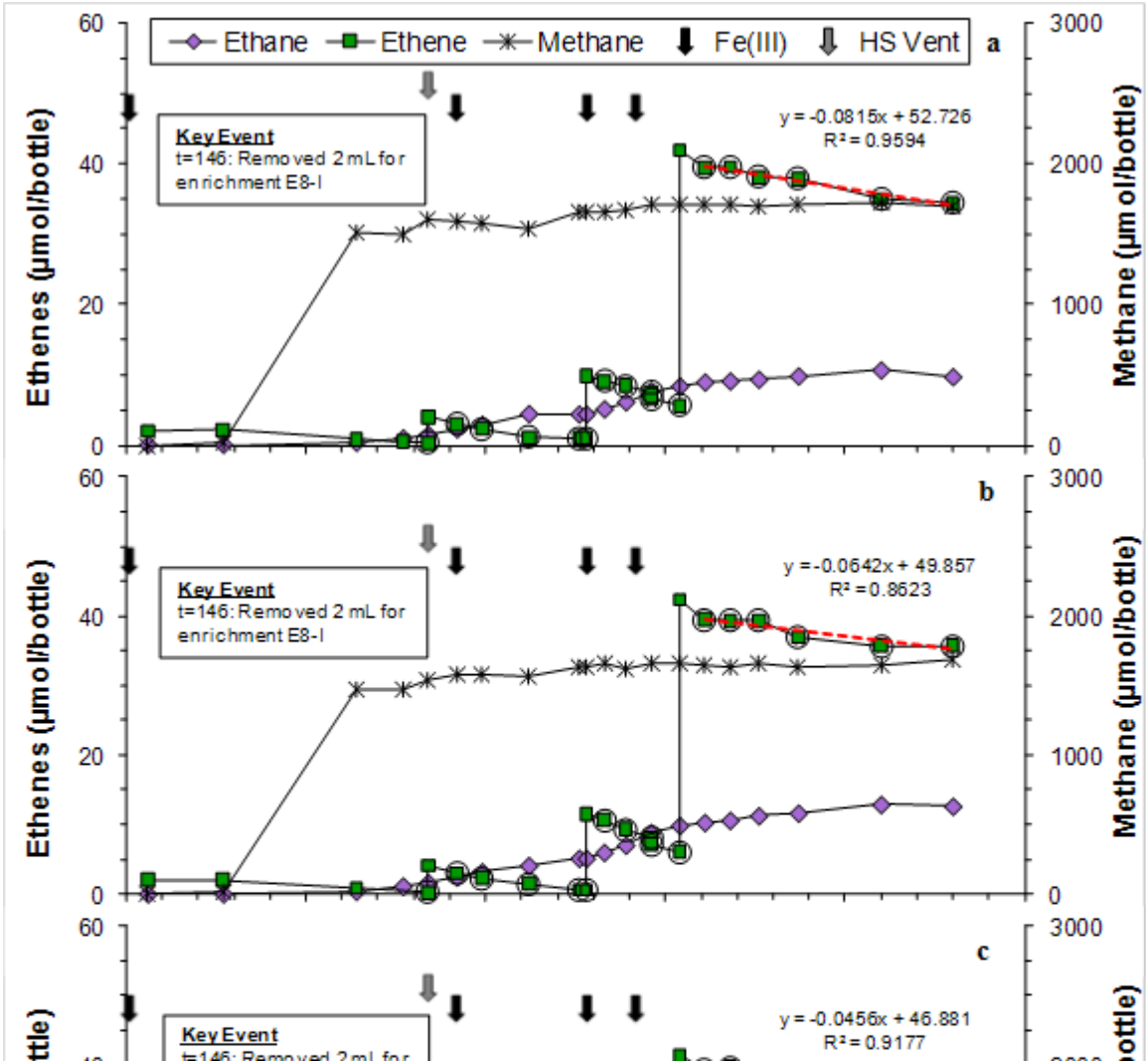


Figure 5.16. Microcosm results for Site #8, Set I, amended with Fe(III), bottle #1 (M8-I-Fe(III)-1) (**a**); bottle #2 (M8-I-Fe(III)-2) (**b**); and bottle #3 (M8-I-Fe(III)-3) (**c**). The circle around symbols for ethene indicate values that were measured with the Carbosieve SII column, rather than the Carbopack B column.

Table 5.1. Summary of the effect of ethane concentration on the percent reduction ratio for microcosms in Site #8, Set I (M8-I), Site #9 (M9) and the enrichment from Site #9 (E9).

Identifier	Terminal Electron Acceptor	Ethene Addition (day)	Initial Measured (µmol/bottle)	% Volume of Ethene/Volume of Headspace	% Reduction Ratio: Ethane/Ethene
M8-I	**Fe(III)**	6	2.1±0.060	0.052±0.0015	84±3
M8-I	**Fe(III)**	84	3.0±0.065	0.073±0.0016	111±4
M8-I	**Fe(III)**	128	10±1.1	0.25±0.027	83±5
M8-I	**Fe(III)**	154	39±0.032	0.98±0.00078	38±24
M8-I	**Fe(III)EDTA**	6	2.1±0.041	0.053±0.0010	88±1
M8-I	**Fe(III)EDTA**	84	2.8±0.079	0.070±0.0020	114±5
M8-I	**Fe(III)EDTA**	128	5.9±1.4	0.15±0.034	-[a]
M8-I	**Fe(III)EDTA**	135	41±2.0	1.0±0.051	6±9
M8-I	**SO_4^{2-}**	6	2.2±0.039	0.054±0.0010	81±12
M8-I	**SO_4^{2-}**	91	4.5±1.1	0.11±0.027	76±16
M8-I	**SO_4^{2-}**	133	7.2±4.0	0.18±0.10	-[a]
M8-I	**SO_4^{2-}**	141	43±1.0	1.1±0.024	-[b]

M9 [c]	**SO_4^{2-}**	584, 621	120±2.4	3.0±0.059	65±13
M9 [d]	**SO_4^{2-}**	720	120±1.3	3.0±0.032	31±4
E9	**SO_4^{2-}**	6	110±1.9	4.5±0.078	1±0.5

[a] Inadequate time interval between sampling points.
[b] No statistically significant decrease in ethene.
[c] Averages for the first 3% addition of ethene to bottles #1 and #2 (which were made on different days).
[d] Averages for the second 3% addition of ethene to bottles #1 and #2.

Monitoring of the Fe(III)-amended microcosms continued through day 762 (data not shown). In two of the three bottles, ethene continued to decrease. The percent loss in ethene attributable to ethane rose to 56-65%; this was still sufficiently below stoichiometric to suggest that some of the decline in ethene was due to an oxidative process. In the third bottle, ethene and ethane remained unchanged between days 280 and 762.

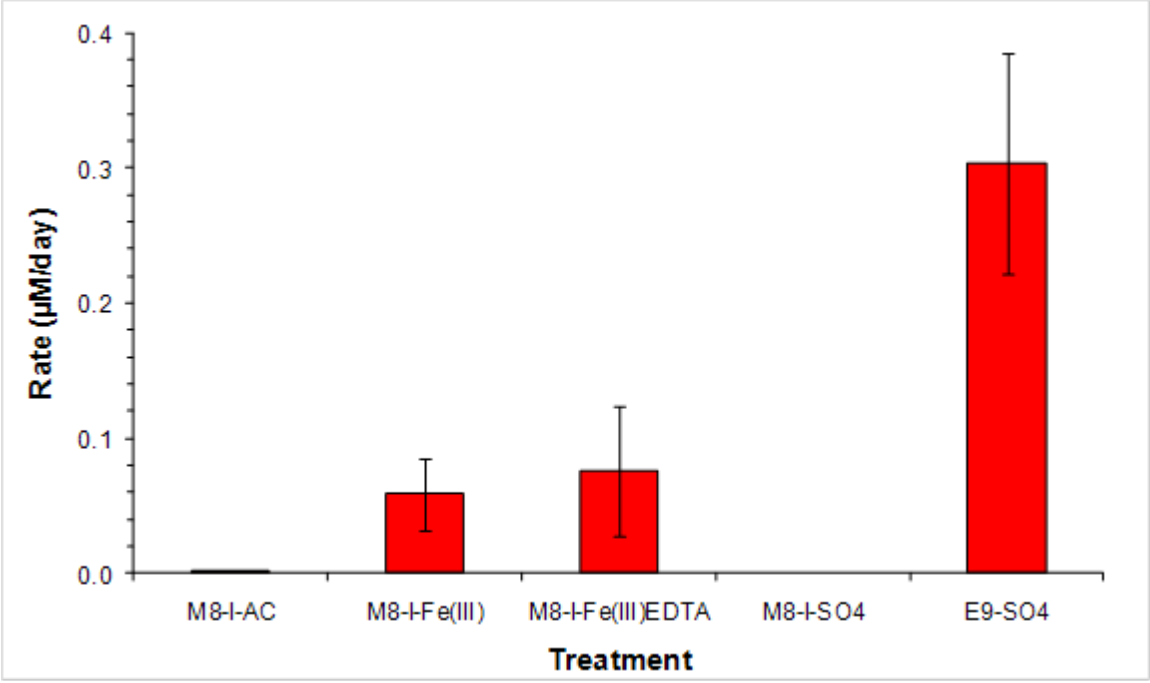


Figure 5.17. Average rates per treatment for microcosms from Site #8, Set I (M8-I) and enrichments from Site #9 (E9). Error bars represent the propagation of error using the standard deviation.

As shown in Figure 5.18, results for ethene in the bottles amended with Fe(III)-EDTA (plus the same level acetate and yeast extract) followed a similar trend to the ones amended with amorphic Fe(III). Ethane was the predominant product from the first two additions of ethene (Table 5.1) and there was a substantial amount of methane formation that required venting on the headspace. The time interval between the third and fourth additions of ethene did not permit determination of ethene to ethane stoichiometry. The fourth addition on day 135 increased the ethene concentration in the headspace to 1.0%. Ethene subsequently decreased at a rate of 0.035-0.092 µmol/bottle/d (from day 161 to 230); only 6% of the decrease in ethene was attributable to an increase in ethane (Table 5.1), suggesting that the balance of ethene consumption was a consequence of some other process, such as oxidation. Over this same interval, there was no statistically significant change in methane (based on a Student's *t*-test for the slope of the regression line for methane data, α=0.05), indicating that the decreases in ethene were not likely due to diffusive losses. Over the full incubation period, five additions of Fe(III) were made, totaling 200 µeeq/bottle (equivalent to 16 mg COD/L). No attempt was made to quantify the amount of Fe(III) that may have been reduced to Fe(II). Using the same procedure described above for the Fe(III)-amended bottles, the rates of ethene decrease for the Fe(III)-EDTA-amended bottles were converted to a net "apparent" rate of oxidation of 0.075±0.048 µM/d (Figure 5.17). This is 27% higher than the average rate for the Fe(III)-amended bottles.

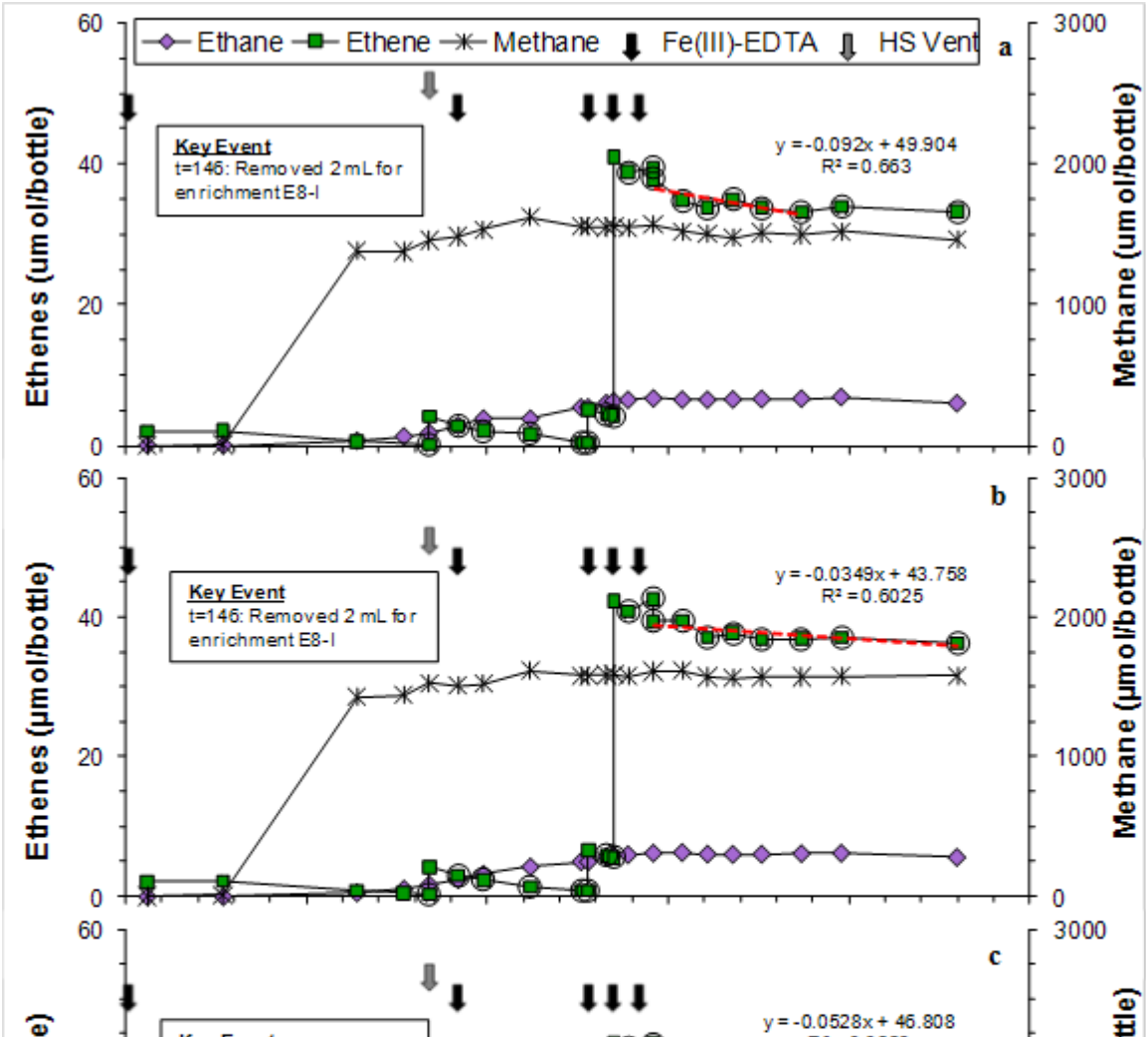


Figure 5.18. Microcosm results for Site #8, Set I, amended with Fe(III)-EDTA, bottle #1 (M8-I-Fe(III)EDTA-1) (**a**); bottle #2 (M8-I-Fe(III)EDTA-2) (**b**); and bottle #3 (M8-I-Fe(III)EDTA-3) (**c**). The circle around symbols for ethene indicate values that were measured with the Carbosieve SII column, rather than the Carbopack B column.

Monitoring of the Fe(III)-EDTA amended microcosms continued through day 762 (data not shown). Ethene continued to decrease without a stoichiometric increase in ethane, although the ratio of ethane to ethene increased to as much as 56. This was still sufficiently below stoichiometric to suggest that some of the decline in ethene was due to an oxidative process.

The microcosms amended with sulfate (along with yeast extract, but no acetate) followed the same initial trend; i.e., the first two additions of ethene were predominantly reduced to ethane (Table 5.1). However, after the ethene level was increased to 1.0% with the fourth addition, there was no statistically significant decrease in ethene through day 230, either by reduction to ethane or oxidation. Consequently, the apparent oxidation rate for the sulfate-amended treatment is shown as zero on Figure 5.17. A total of 1.3 mM of sulfate was consumed.

In the glucose and GLF-amended treatments, ethene consumption occurred only via reduction to ethane; there was no indication of ethene oxidation. Methane output was very high in both treatments, as a consequence of the high level of electron donor added (13.7-16.9 g/L COD). The rate of ethene reduction was slower than in the other treatments.

In summary, ethene was consumed in all of the anaerobic Site #8, Set I-A microcosms. When ethene levels were below 1% of the headspace, consumption of ethene occurred primarily by reduction to ethane, with molar recoveries averaging above 90% (Table 5.1). However, when ethene levels were increased to 1% of the headspace volume in the two treatments with Fe(III) added, there was a statistically significant rate of decrease in ethene without a stoichiometric increase in ethane. During this interval, a lack of change in methane levels suggested that the decreases in ethene were not a consequence of diffusive losses. However, since [^{14}C]ethene was

not added to these bottles, it was not possible to determine what products may have formed other than ethane. For this reason, a second set was prepared and [^{14}C]ethene was added.

5.8.2 Site #8, Set II

Set II was prepared two months after Set I. The same eight treatments described for Set I were prepared, with the main difference being the addition of [^{14}C]ethene Three additional treatments were also prepared: 1) VC under aerobic conditions; 2) ethene under anaerobic conditions, with no TEA added; and 3) VC amended with Fe(III), to test for VC bio-oxidation under iron-reducing conditions.

As expected, ethene was completely consumed without a lag in the live treatment with oxygen added, while there was no statistically significant loss of ethene from the autoclaved controls during 105 days of incubation (data not shown). VC was also completely consumed in a separate set of live aerobic bottles over the same time period. This was notable, since the soil had no prior history of exposure to chlorinated organic compounds. It was also notable that there was no detectable lag period prior to the onset of VC consumption, and a second addition of VC was consumed at a higher rate (data not shown).

Over 122 days of incubation, there was no significant decrease in ethene from either the unamended anaerobic microcosms or the nitrate-amended microcosms; the nitrate added at time zero (0.15 mM) was consumed.

The Fe(III) amended bottles were prepared with a medium that included 2.7 g/L of sodium acetate and 50 mg/L of yeast extract. Three or four additions of Fe(III) were made during the first 119 days of incubation. The first addition of ethene was consumed by day 82, with 92% recovery as ethane. During this period, methane output increased rapidly and reached a plateau. The second addition of ethene on day 82 increased the headspace concentration to approximately 1% (40 µmol/bottle). At the same time, [^{14}C]ethene was added (accompanied by trace levels of toluene from the ^{14}C stock solution, as explained by Reid (2010). At the end of the incubation period (day 531), ethene had decreased by only 8% and this was matched by a comparable increase in ethane. The microcosms were then sacrificed for ^{14}C analysis. Less than 1% of the [^{14}C]ethene added was recovered as $^{14}CO_2$ or [^{14}C]NSR, indicating there was no anaerobic oxidation of ethene. Since the focus of the ^{14}C measurements was on bio-oxidation, no attempt was made to quantify the amount of [^{14}C]ethane formed.

In the microcosms amended with Fe(III) and VC instead of ethene, there was no significant decrease in VC over the 122 days of incubation.

The Fe(III)-EDTA amended microcosms contained the same medium as the ones with Fe(III). Two additions of Fe(III)-EDTA were made during the first 60 days of incubation. The first ethene addition was consumed by day 102, with 92% recovery of ethane. The second addition of ethene on day 102 increased the headspace concentration to approximately 1% (40 µmol/bottle). At the same time, [^{14}C]ethene was added (accompanied by a trace level of toluene). At the end of the incubation period (day 531), the 9% decrease in ethene was matched by a stoichiometric increase in ethane. On day 531, the microcosms were sacrificed for ^{14}C analysis. Less than 1%

of the [^{14}C]ethene added was recovered as $^{14}CO_2$ or [^{14}C]NSR, indicating there was no anaerobic oxidation of ethene in this treatment. [^{14}C]ethane was not quantified.

In the microcosms amended with sulfate, the only electron donor in the medium was yeast extract (ca. 75 mg/L COD). In two of the three microcosm, 90-100% of the ethene consumption was attributable to ethane formation; the third bottle broke early in the incubation period. [^{14}C]ethene was added on day 102. On day 531, the microcosms were sacrificed for ^{14}C analysis. Less than 2% of the [^{14}C]ethene added was recovered as $^{14}CO_2$ or [^{14}C]NSR, indicating there was no anaerobic oxidation of ethene in this treatment. [^{14}C]ethane was not quantified.

In the glucose-amended microcosms, ethene consumption also corresponded to ethane accumulation. [^{14}C]ethene was added on day 102. A second addition of ethene on day 207 increased the headspace concentration to approximately 1% (40 µmol/bottle). There was little to no decrease in ethene thereafter. On day 531, the microcosms were sacrificed for ^{14}C analysis. Less than 2% of the [^{14}C]ethene added was recovered as $^{14}CO_2$ or [^{14}C]NSR, indicating there was no anaerobic oxidation of ethene in this treatment. [^{14}C]ethane was not quantified.

The microcosms amended with GLF were incubated for only 122 days and were not spiked with [^{14}C]ethene. There was no evidence of ethene degradation during the incubation period, although an appreciable amount of methane accumulated. This is consistent with the high level of biodegradable COD added.

5.8.3 Enrichments from Site #8, Set I

Based on the initially promising results for anaerobic oxidation of ethene in the Set I microcosms amended with Fe(III) (Figure 5.16), Fe(III)-EDTA (Figure 5.18) and sulfate, a set of enrichments was prepared with 2 mL of well-mixed samples from the microcosms (plus 98 mL of either the Fe(III)-2 or sulfate medium (Table 2.1). A higher initial level of ethene was used in the enrichments than the microcosms (0.5% headspace concentration). The terminal electron acceptors were added only at the start of the incubation period. Following 84 days of incubation, there was no significant decrease in ethene, either by reduction to ethane or an oxidative process.

5.9 SITE #9

Site #9 is the Twin Lakes area at the Savannah River Site, where a plume of TCE discharges into a wetland and undergoes reductive dechlorination to ethene and ethane, at pH levels in the range of 5 to 6. Given the on-going flow of VC and ethene through the wetland, it was considered a candidate for microbes capable of anaerobic oxidation. Two sets of microcosms were prepared, along with one set of enrichments.

5.9.1 Site #9, Set I

This set of microcosms was prepared by Eaddy (2008) prior to the start of this project. Monitoring for this project started on day 500 (Figure 5.19). In bottle #1, the amount of ethene added on day 554 was approximately 1% of the headspace volume (i.e., 40 µmol/bottle); a lower amount of VC (6.2 µmol/bottle) was added at the same time. Over the next 30 days, all of the

VC was dechlorinated and ethene decreased by 29 µmol/bottle, with 90% recovery as ethane. During this interval, methane output rose in response to three additions of lactate (days 535, 554 and 573), which was the only electron donor provided to these microcosms. On day 584, more VC and ethene were added, so that the headspace concentration of ethene was approximately 3%. Between days 584 and 720, all of the VC was consumed along with nearly all of the ethene, 52% of which was recovered as ethane. The lower extent of ethane recovery suggested that one half of the ethene may have undergone anaerobic bio-oxidation. During this interval, sulfate was added twice (totaling 36 mM); however, sulfate measurements were not made. There was no new production of methane after day 584. A third high dose of ethene was added on day 720. Between days 727 and 750, ethene decreased at a slow rate, with 34% recovery as ethane. Thereafter, there was no significant decrease in ethene.

Bottle #2 behaved similarly. After increasing the ethene dose to 3% of the headspace, 78% of ethene consumed was recovered as ethane (Table 5.1). Although less than stoichiometric recovery of ethane suggests some ethene consumption may have occurred via anaerobic oxidation, it is not possible to conclude this with confidence in the absence of daughter products other than ethane.

5.9.2 Site #9, Set II

The intent of preparing an additional set of microcosms with soil and groundwater from the Twin Lakes wetland at SRS was to determine if the results of Johnson (2009) could be replicated, with respect to favoring ethene conversion via a pathway other than reduction.

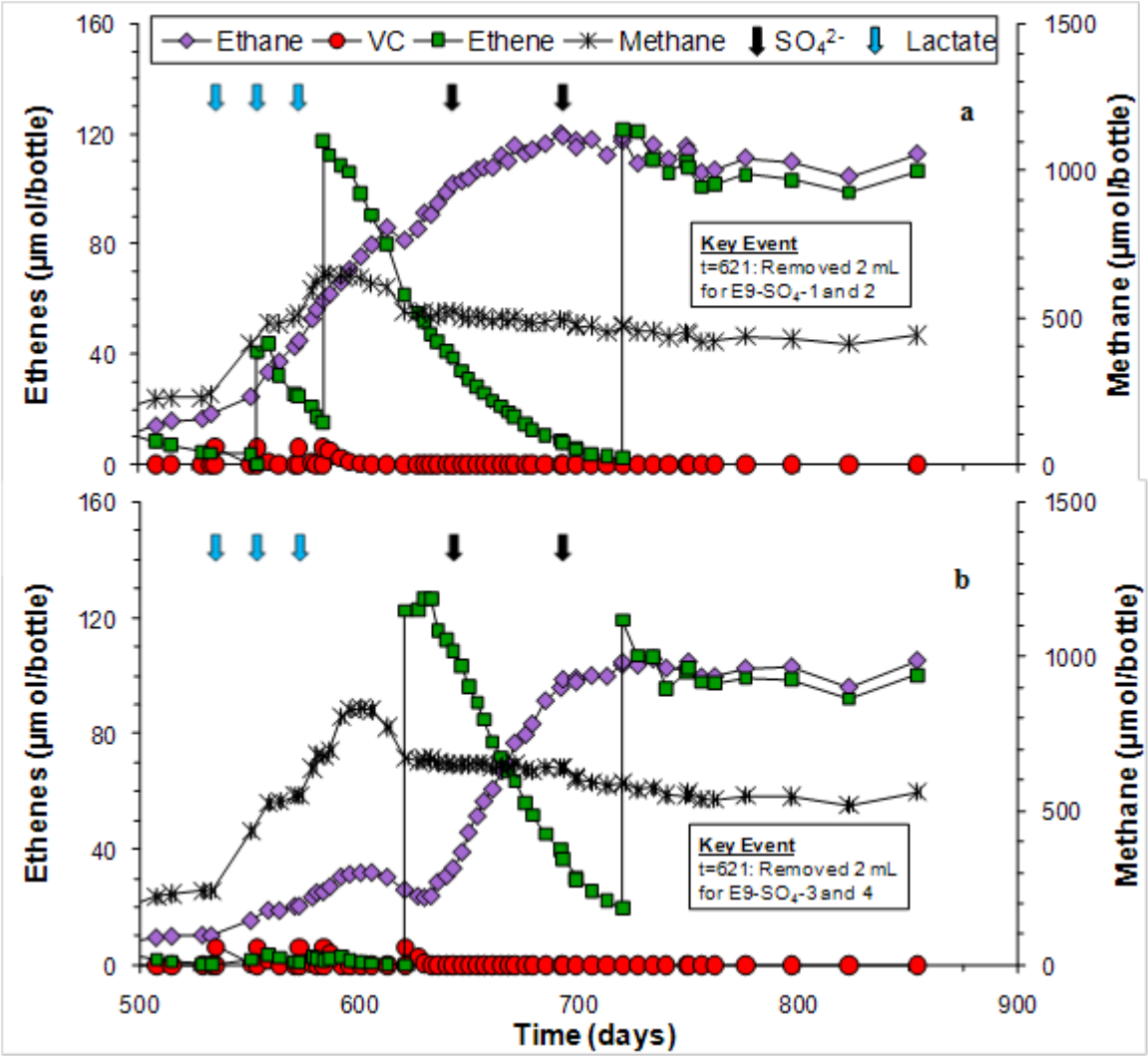


Figure 5.19. Microcosm results for Site #9, Set I, amended with sulfate, for **a)** bottle #1 (M9-SO_4-1) and **b)** bottle #2 (M9-SO_4-2).

Treatments with VC were also included, as well as six electron acceptor amendments. Including the autoclaved controls, a total of 96 microcosms were constructed (Tables 2.2 and 2.3). Average results for the triplicate unamended bottles that received only VC are shown in Figure 5.20a. Five additions of VC were reductively dechlorinated to ethene, most of which was then reduced ethane. Methane was produced throughout the 246 days of incubation. These results indicated that the soil and groundwater provided an excess of electron donor needed for reduction, to such an extent that a significant amount of methane was also produced. The treatment that received VC and ethene is shown in Figure 5.20b. In spite of the considerably higher level of ethene compared to VC, these microcosms reduced VC to ethene at a similar rate in comparison to the treatment with only VC added. Ethene reduction to ethane started at approximately the same time and most of the ethene (formed plus added) was reduced to ethane by the time monitoring stopped on day 246. Methanogenesis was delayed somewhat by the high initial concentration of ethene; as it declined, methane output increased.

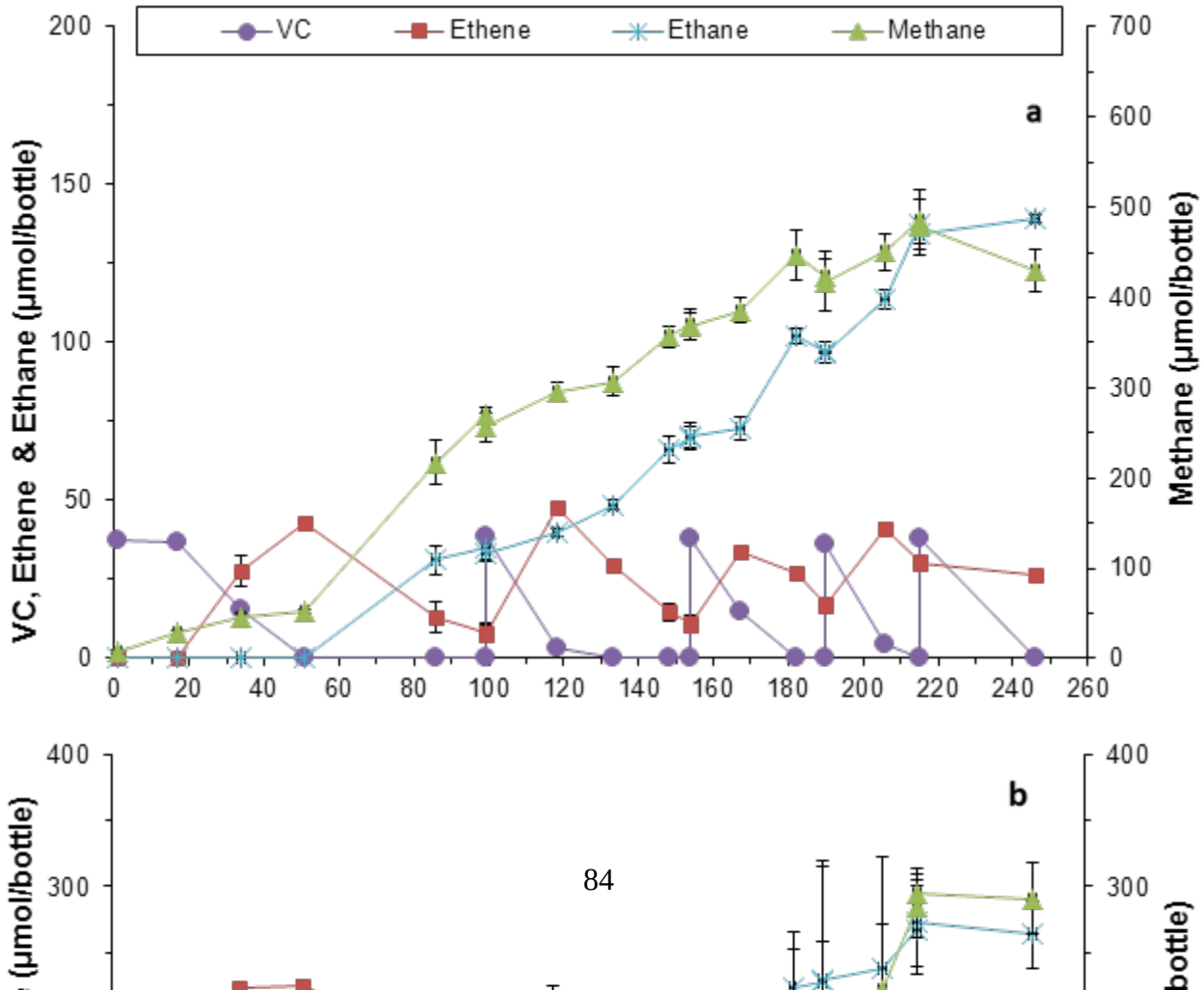


Figure 5.20. Site #9, Set II microcosm results for the unamended treatment, average of triplicate bottles for **a)** VC added (Unamended-VC); and **b)** VC + ethene added (Unamended-VC-ETH). Error bars represent one standard deviation.

For comparison, there were no significant losses of VC, ethene or ethane over 237 days of incubation from the autoclaved controls (data not shown). The results confirmed that grey butyl rubber septa are effective in preventing significant diffusive losses of VC, ethene and ethane.

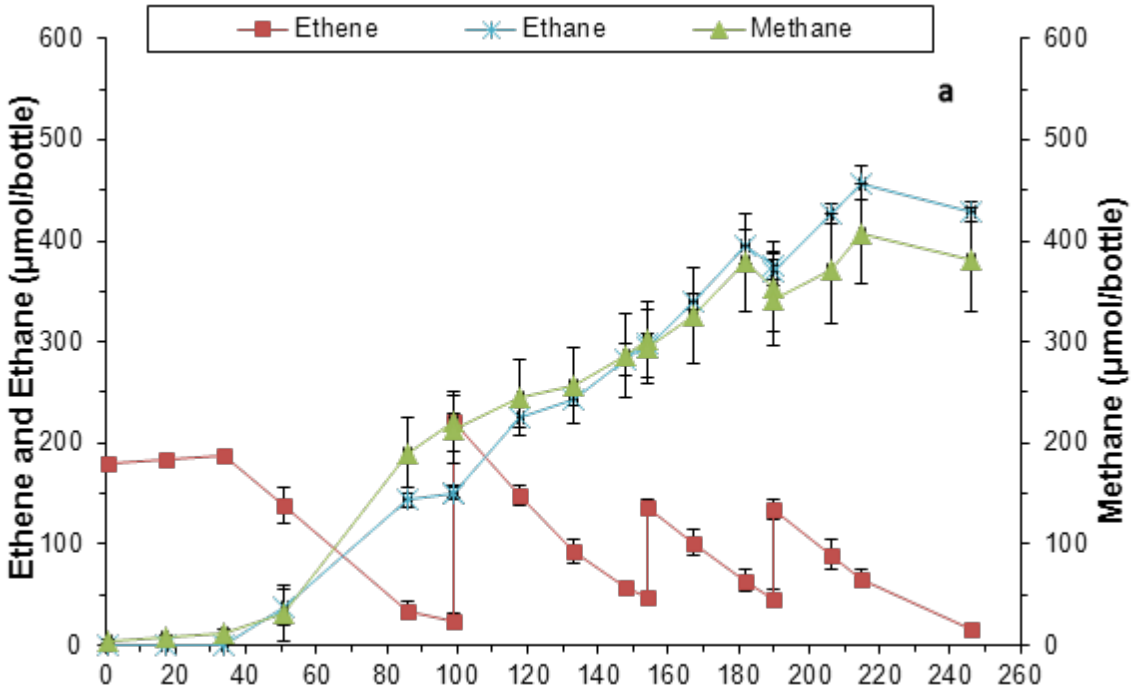
Average results for the triplicate unamended bottles that received only ethene are shown in Figure 5.21a. Four additions of ethene were reduced to ethane. Methane output started as the first addition of ethene decreased. The treatment that received ethene and ethane is shown in Figure 5.21b. The presence of ethane did not deter the amount of ethene that was reduced to ethane. Methane output followed the same pattern as in the treatment with only ethene added.

As these results indicate, none of the unamended treatments exhibited any evidence for anaerobic oxidation of VC or ethene. Likewise, there was no indication of oxidative activity in any of the treatments with various electron acceptors added (data not shown). As in the unamended treatments, all of the VC that was consumed was reduced to ethene and/or ethane; all of the ethene that was consumed was reduced to ethane.

A summary of the amounts of VC and ethene consumed by treatment is shown in Figure 5.22. These results indicate that addition of an electron acceptor significantly reduced the amount of VC and ethene consumed, with the impact on ethene greater than on VC. In five of the six treatments with an electron acceptor added (nitrate, Fe(III)-EDTA, AQDS, Fe(III)+AQDS, and sulfate), the presence of ethene along with VC significantly decreased the amount of VC consumed (Figure 5.22a). Nevertheless, this did not lead to an onset of VC oxidation. With ethene, the presence of an electron acceptor had such a dampening effect on ethene reduction that having ethane present had no impact on the amount of ethene consumed (Figure 5.22b).

Figure 5.23a summarizes the cumulative amount of electron acceptor added, expressed in meq per bottle (5 meq/mmol NO_3^-; 1 meq/mmol Fe(III); 2 meq/mmol AQDS; 8 meq/mmol SO_4^{2-}). Measurements of nitrate, sulfate and Fe(II) were made periodically (data not shown); when the results indicated that nitrate, sulfate, or Fe(III) was depleted, more was added. Overall, at 66-90% of the nitrate added was consumed; 53-69% of the AQDS added was reduced to AH_2QDS; and 54-68% of the sulfate added was consumed. With the Fe(III) treatments, the amount of Fe(II) that accumulated was more variable, ranging from 4.9-11% of the Fe(III) added. Addition of electron acceptor significantly decreased methane output (Figure 5.23b), which is another indication that the electron acceptors were being utilized.

In summary, there was no indication of anaerobic VC or ethene oxidation in the SRS microcosms. Adding ethene to the VC microcosms decreased VC reduction to ethene, but did not initiate VC oxidation. Ethene reduction to ethane was strongly inhibited by addition of nitrate, Fe(III), AQDS, and sulfate.



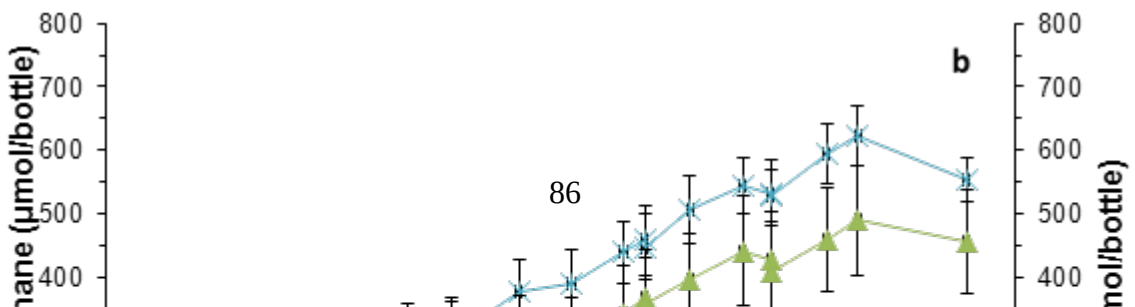


Figure 5.21. Site #9, Set II microcosm results for the unamended treatment, average of triplicate bottles for **a)** ethene added (Unamended-ETH); and **b)** ethene + ethane added (Unamended- ETH-ETA). Error bars represent one standard deviation.

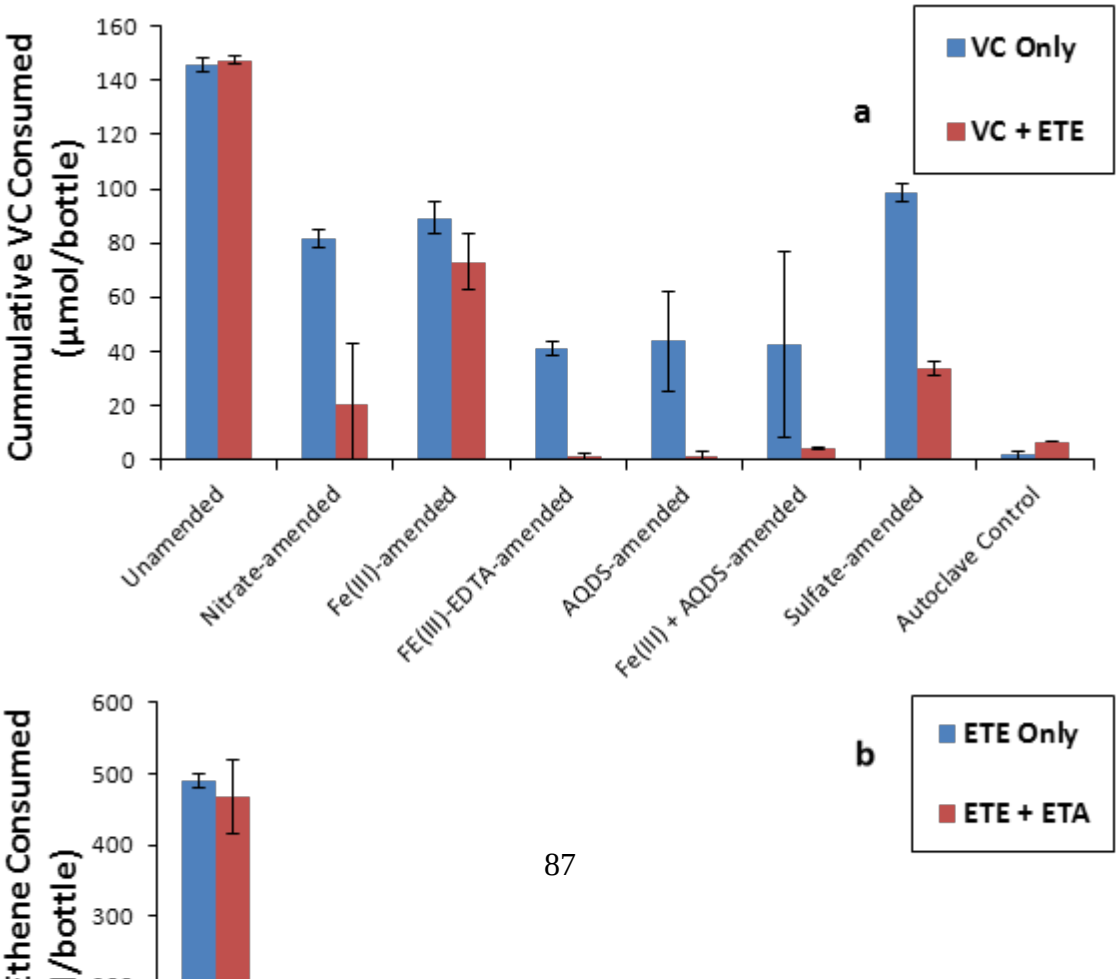


Figure 5.22. Summary of results for the Site #9, Set II microcosms in terms of **a)** total VC consumed; and **b)** total ethene consumed.

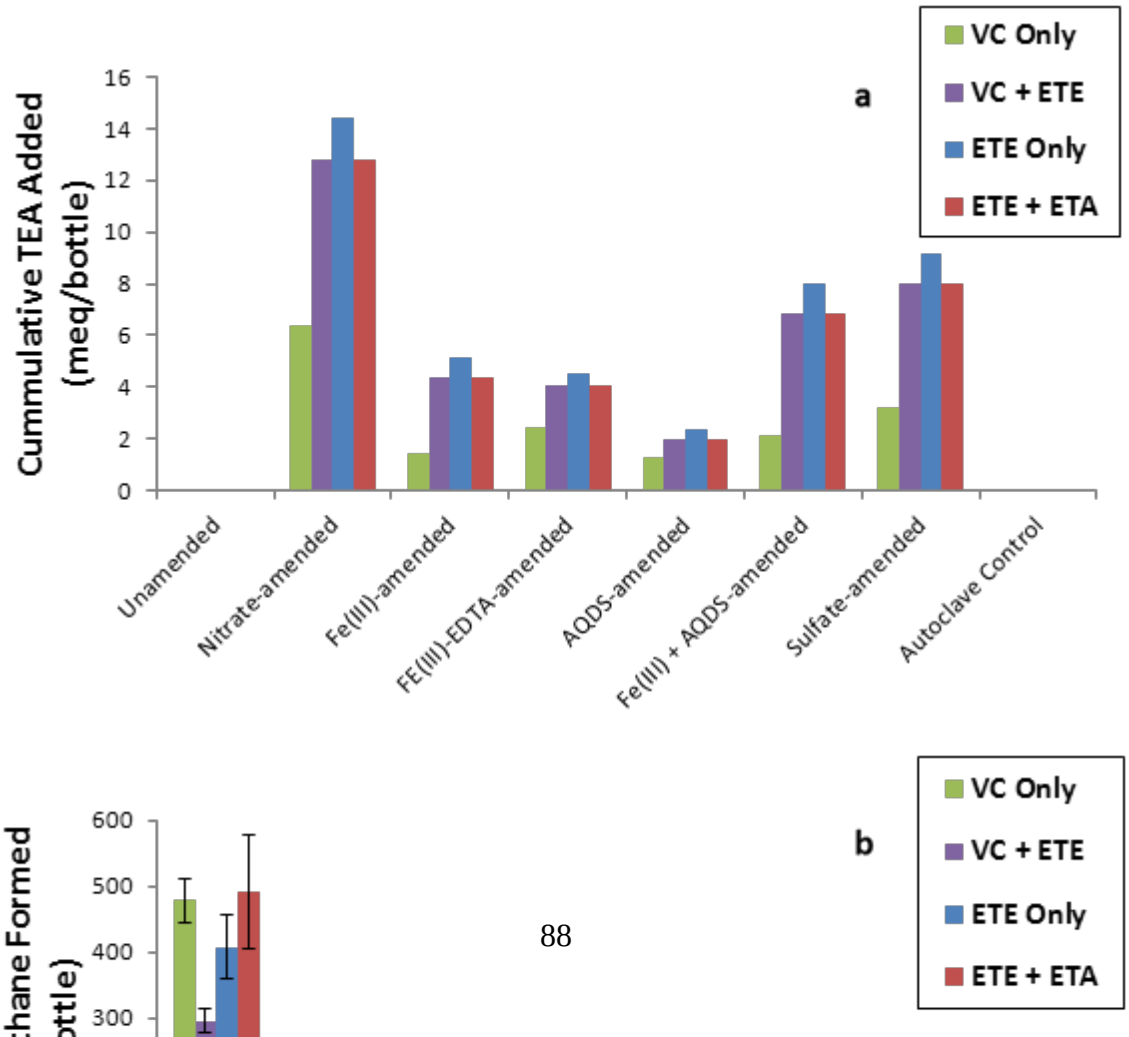


Figure 5.23. Summary of results for the Site #9, Set II microcosms in terms of **a)** total terminal electron acceptor added; and **b)** total methane produced.

5.9.3 Enrichments from Site #9, Set I

This set of enrichments was prepared with 1 mL of well-mixed samples from the Site #9 Set I microcosms plus 99 mL of the MSM medium (Table 2.1). The motivation for preparing these bottles was the suggestion of ethene bio-oxidation in the microcosms based on less than stoichiometric accumulation of ethane from the ethene consumed (Figure 5.19). A high initial dose of ethene (approximately 5% of the headspace volume) was provided (Table 2.3), with the intent of minimizing ethene reduction to ethane. Three medium controls (i.e., 100 mL of MSM) were prepared along with quadruplet live bottles.

Over the first 79 days, ethene decreased in the live bottles at a rate of 0.34 µmol/bottle/day, with no significant accumulation of ethane. This compares to an ethene consumption rate of 0.11 µmol/bottle/day in the MSM controls over 175 days of incubation period. The net rate of ethene consumption in the live bottles was 0.30±0.082 µM/day, obtained by subtracting the controls from the live bottles and converting µmol/bottle/d to µM/d using equation 2.1. This net rate is approximately three times higher than the apparent oxidation rates estimated for the M8-I-Fe(III) and M8-I-Fe(III)EDTA treatments (Figure 5.17). During the first 99 days of incubation, 0.80±0.11 mM of sulfate was consumed in the live bottles but not significantly in the controls (Figure 5.19b), suggesting that ethene consumption correlated with sulfate reduction. Electron donor either came from oxidation of the ethene or carryover of organics in the inoculum. The next time sulfate was measured (day 128), there was no additional sulfate consumption (the sulfate level was actually higher, reflecting a degree of measurement error); there was also no significant change in the medium control. The cessation in sulfate consumption was consistent

with the cessation in a biotic decrease in ethene. Methane output was less than 0.6 µmol/bottle over this interval.

To obtain additional evidence of oxidative activity, [^{14}C]ethene was added on day 79 day. On day 891, the bottles were sacrificed for analysis of ^{14}C. In three of the four bottles, less than 2% of the [^{14}C]ethene added was recovered as $^{14}CO_2$ or [^{14}C]NSR, indicating there was no anaerobic oxidation of ethene. However, in a fourth bottle (E9-SO4-4), 8.7% of the ^{14}C was recovered as $^{14}CO_2$ and 6.2% as [^{14}C]NSR. While this indicated that bio-oxidation of ethene occurred, the results were equivocal since ca. 70% of the ^{14}C was lost during incubation.

5.10 SITE #10

At Site #10, the groundwater is contaminated predominantly with PCE. A portion of the contaminant plume discharges to a wetland. Hickey (2010) prepared microcosms with soil and groundwater from the site and observed that PCE was dechlorinated to ethene, which was further reduced to ethane. For the purpose of this project, the microcosms were used as a source of inoculum to develop six enrichment bottles in MSM (Table 2.1). Three of the bottles received only unlabeled ethene; the other three received ethene plus [^{14}C]ethene. The initial amount of ethene added was 12 µmol/bottle, along with lactate (0.11 mM).

Over the first 45 days, ethene decreased by 51%, with 91% recovery as ethane. Methane output increased in parallel with ethene reduction. [^{14}C]ethene was added on day 45, along with enough unlabeled ethene to raise the ethene level to 1.1% of the headspace. Between days 45 and 475, there was a 22% decrease in ethene, with 93% recovery as ethane. On day 475, the microcosms were sacrificed for ^{14}C analysis. Less than 1% of the [^{14}C]ethene added was recovered as $^{14}CO_2$ or [^{14}C]NSR, indicating there was no anaerobic oxidation of ethene in this treatment. No attempt was made to quantify the amount of [^{14}C]ethane formed.

The triplicate bottles that did not receive [^{14}C]ethene behaved similarly. In summary, there was no evidence of ethene bio-oxidation in the Site #10 enrichment bottles, even after increasing ethene to a headspace concentration that was expected to inhibit ethene reduction to ethane.

5.11 EVALUATION OF VC OXIDATION WITH OXYGEN PLUS NITRATE

Bio-oxidation of VC under aerobic conditions involves two uses for oxygen: as a reactant in the first step for an alkene monooxygenase (one mole of O_2/mol VC); and as the terminal electron acceptor. The intent of this experiment was to determine if the use of oxygen as an electron acceptor could be replaced by nitrate, with use of oxygen as a reactant still occurring. This is a scenario that could be envisioned when the availability of oxygen is limited and nitrate is in excess.

To evaluate this hypothesis, 12 enrichment bottles were prepared with inoculum from Site #7, Sets I-A and I-B microcosms that received oxygen (to serve as a positive control) and rapidly consumed VC. Samples from these microcosms were used to inoculate three serum bottles (1.5% v/v) along with the O_2/NO_3^- medium (Table 2.1). These were provided with repeat additions of VC under aerobic conditions, with the intent of further enriching for aerobic oxidation of VC. Next, the contents of the three bottles were mixed and aliquots were used to prepare 12 new bottles (also at 1.5% v/v inoculum, with the same medium), consisting of four

treatments (in triplicate): 1) VC added (20 µmol/bottle) with an excess oxygen (81 µmol/bottle), to ensure that aerobic conditions were maintained and to serve as a positive control for VC oxidation; 2) VC added (20 µmol/bottle) with less the stoichiometric amount of oxygen needed (16 µmol/bottle), to determine if VC oxidation would stop when oxygen levels decreased close to or below detection; 3) VC added (20 µmol/bottle) with less than the stoichiometric amount of oxygen needed (16 µmol/bottle), plus an excess of nitrate added (38 µmol/bottle) to replace oxygen as the terminal electron acceptor; and 4) Acetate (64 µmol/bottle) and nitrate added (96 µmol/bottle), to serve as a positive control for the presence of denitrifying microbes (i.e., since acetate is a common substrate for denitrification).

As expected, all of the positive controls exhibited aerobic biodegradation of VC. The first addition of VC was consumed within 28 days, with a stoichiometry of 1.9 µmol O_2/µmol VC. Biodegradation of a second addition of VC proceeded at a similar rate through day 76, when the oxygen level decreased to 10 µmol/bottle or less. The VC level did not change between day 76 and 101; on day 101, an excess of oxygen was added for a second time. VC biodegradation resumed and reached the detection limit on day 108. These results confirmed the presence of aerobic VC oxidizing bacteria that have an oxygen threshold in the vicinity of 10 µmol/bottle. Using equation 2.1 and a Henry's Law constant for oxygen of 31.676 (mg/L gas per mg/L water) (Gossett, 2010), this equates to an aqueous phase concentration of 0.1 mg/L.

In the treatment provided with a limited amount of oxygen, VC and oxygen declined initially, until oxygen reached approximately 10 µmol/bottle. Thereafter, there was no significant decrease in VC. These results were consistent for the first treatment, i.e., that the oxygen threshold for VC biodegradation was approximately 10 µmol/bottle.

In the treatment provided with a limited amount of oxygen but an excess of nitrate, there was an initial decrease in VC until the oxygen reached approximately 10 µmol/bottle. The 225 µM of nitrate that was initially added was consumed by day 28-46 and more was added on day 46. However, VC levels did not decrease during the period when nitrate was available in excess but oxygen was in limited supply. On day 108, 12 µmol of oxygen was added; in response, there was a decrease in VC until oxygen once again plateaued at approximately 5-10 µmol/bottle.

The fourth treatment was used to determine if nitrate reducing microbes were present in the enrichment culture, using acetate as the electron donor. The first addition of acetate and nitrate were consumed by day 27. Both were added a second time on day 45 and once again, both were consumed by day 65. This time, nitrate was added without acetate and the nitrate remained at the same level through day 94. This indicated that no other electron donors were available for nitrate reduction. After adding more acetate on day 94, both the acetate and nitrate were consumed concurrently by day 100. Next, three additions of acetate were made without nitrate. Between days 115 and 147, when the acetate concentration was approximately 2.1 mM, none was consumed in the absence of nitrate. These results confirmed that the enrichment cultures contained denitrifying microbes and that consumption of nitrate and acetate depended on both being present. Furthermore, the results indicated that the lack of VC biodegradation in the treatment with limited oxygen but an excess of nitrate was not a consequence of the enrichment culture lacking denitrifying microbes. Instead, the results suggest that nitrate cannot substitute for oxygen as the terminal electron acceptor even when oxygen is available for the alkene monooxygenase to initiate VC metabolism.

6.0 RESULTS AND DISCUSSION: AEROBIC BIODEGRADATION OF VC

6.1 EXAMINATION OF TERMINAL ELECTRON ACCEPTORS

In one of the triplicate microcosms prepared with soil and groundwater from near the source area of the southern California industrial site, dichloromethane (ca. 180 µM) was consumed within 40 days (without accumulation of chloromethane or methane), while VC consumption (ca. 8.7 µM) occurred between days 230 and 310, without accumulation of ethene. There was no significant decrease of TCE or cDCE during 390 days of incubation in comparison to autoclaved controls (Appendix A-2).

Samples from the microcosm (0.5 mL) exhibiting activity on VC were added to groundwater (99.5 mL) to prepare transfer #1 bottles. The initial dose of VC was consumed in 43 to 77 days (Figure 6.1A). Repeat additions of VC were consumed at a faster rate, without accumulation of ethene, until activity stopped beyond day 140. Samples from these bottles (10 mL) were added to groundwater (90 mL) to prepare transfer #2. The lag time for consumption of the first dose of VC was shorter and a high rate of VC consumption was sustained through day 84 (Figure 6.1B). The electron acceptor associated with VC oxidation was not identified. The site groundwater contained approximately 10.5 mg/L sulfate, <0.1 mg/L nitrate and 2.3 mg/L Fe(II) making sulfate the most likely potential anaerobic electron acceptor. There was no accumulation of methane. Stalled, unamended groundwater microcosms could be stimulated with the addition of fresh groundwater, suggesting that the lacking nutrient or electron acceptor was present within the groundwater (data not presented) (Cline, 2003).

Material from the transfer #2 microcosms was used as inoculum into defined medium for enrichment cultures. These cultures received nitrate (5 mM), nitrite (5 mM), sulfate (5 mM), Fe(III) oxide (5 mM), oxygen (3.7%), or no added terminal electron acceptor. A key difference between the enrichment culture bottles and the transfer #1 and #2 bottles was the use of butyl rubber stoppers with the enrichment bottles, instead of Teflon-coated red rubber ones. VC oxidation activity was compared with abiotic controls to account for loss due to sampling and any abiotic reactions.

In the nitrate or sulfate amended and unamended anaerobic enrichment cultures, no oxidation of VC was observed relative to an abiotic control (data not shown). VC loss was only observed in enrichment cultures that received oxygen. Material from the microaerobic enrichment cultures was used to inoculate defined growth medium and these cultures maintained activity through two 1% transfers in liquid medium (Figure 6.2). These cultures also showed VC oxidation at increasing rates, indicative of growth. Using material from these liquid enrichment cultures, semisolid medium plates were inoculated by streaking for isolation and were incubated microaerobically.

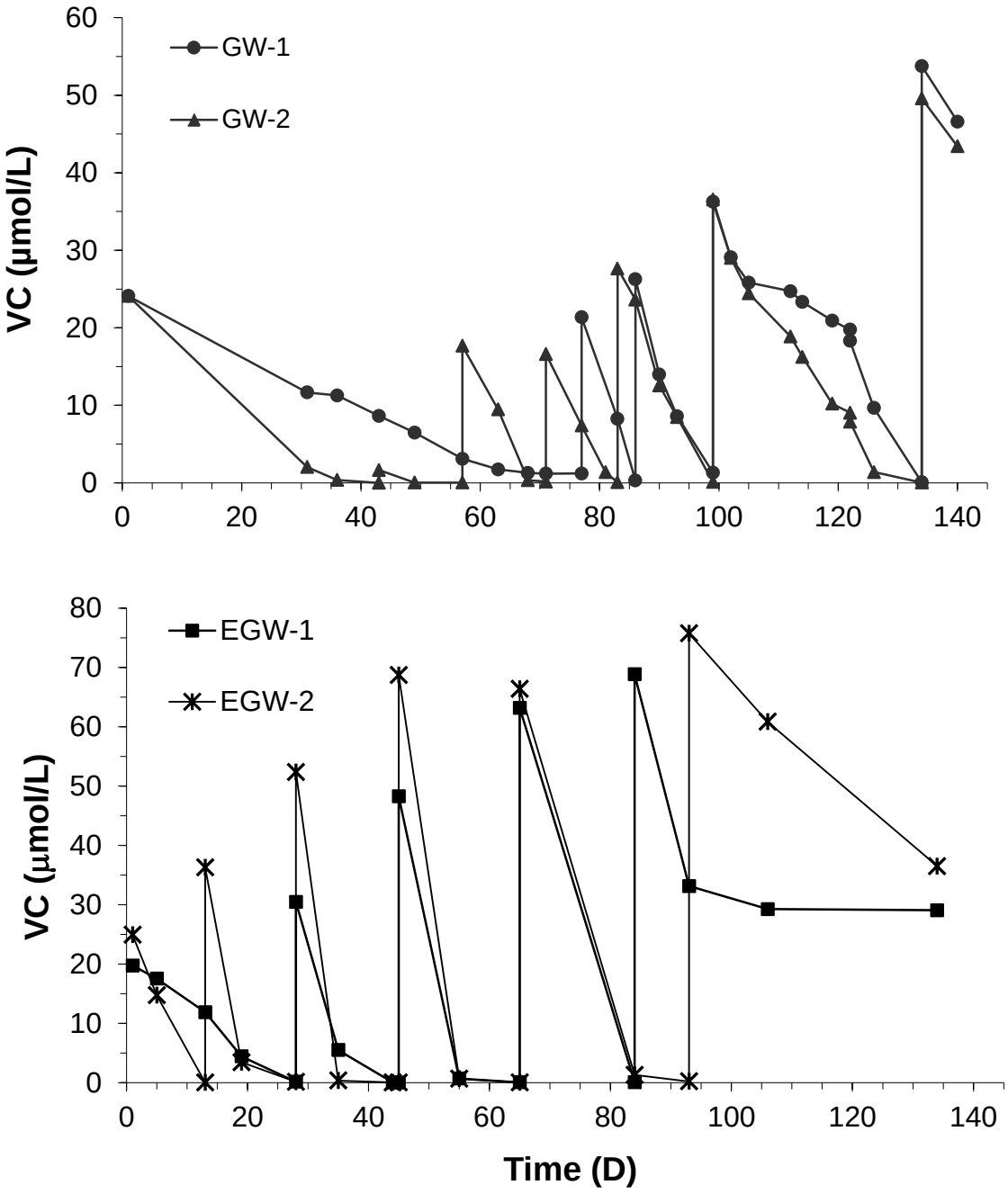


Figure 6.1. VC oxidation in unamended anaerobic microcosms for (A) transfer #1 (GW) and (B) transfer #2 (EGW). Vertical lines indicate additions of VC.

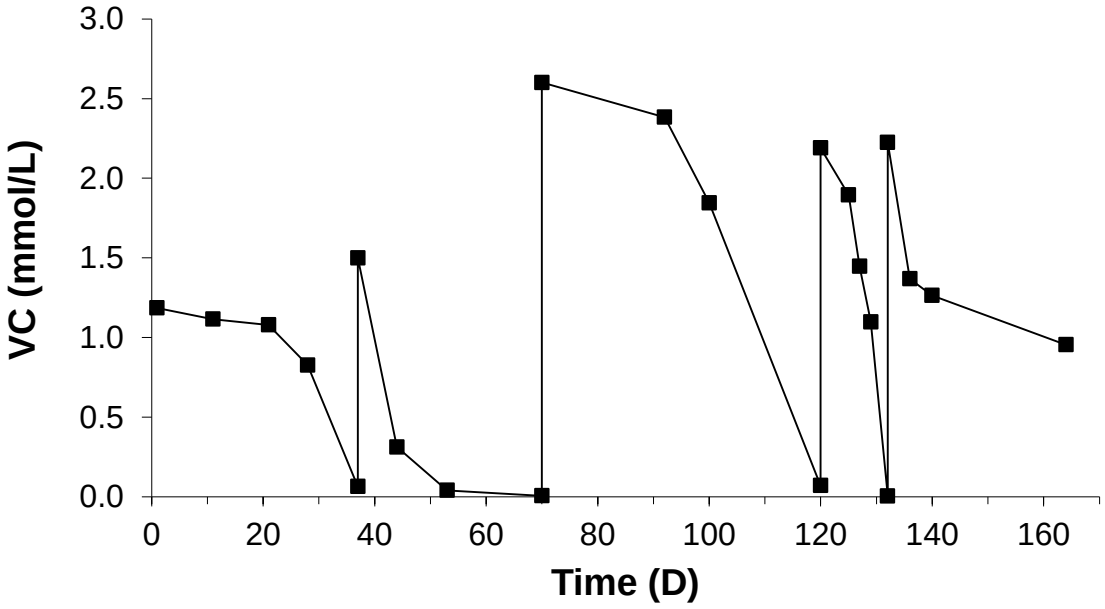


Figure 6.2. Second-generation enrichment culture, inoculated from groundwater microcosms. Disappearance of VC is indicative of its oxidation under microaerobic conditions. Vertical lines indicate additions of VC.

After 23 days, flat brownish colonies, ca. 1 mm in diameter, were visible. Microscopic observation showed organisms from the colonies were small Gram-positive rods. Growth in liquid medium inoculated from a colony was slow to begin, taking 86 days to consume the first dose of VC (data not shown). The second dose of VC was consumed within another 12 days. Other organisms capable of growing on VC have been found to show a lag in growth (Verce et al., 2000). For some isolates, this lag has been attributed to the absence of VC-epoxide in cells needed to induce alkene monooxygenase (Mattes et al., 2007).

Other VC oxidizing bacteria are also able to oxidize ethene (Coleman and Spain, 2003). To test if our isolate was able to utilize ethene as well as VC, triplicate tubes were inoculated from a VC grown culture. After 23 days, no VC or ethene remained (Figure 6.3). The consumption of VC and ethene followed an increase in OD concomitant with VC or ethene disappearance. For the second dose of VC or ethene, the consumption rate increased along with an increase in optical density. Although the OD was low, cultures unamended with either VC or ethene failed to show an increase in OD. Thus, the increase in OD represents growth that is dependent on VC or ethene.

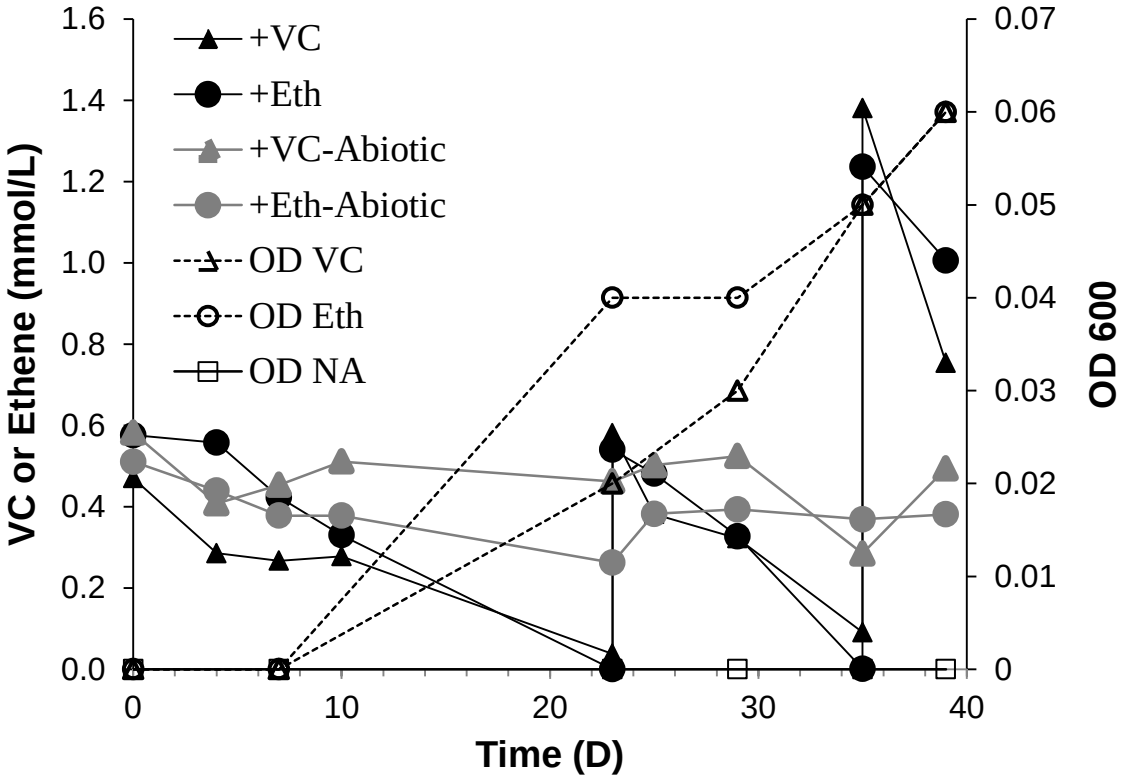


Figure 6.3. Growth of the VC oxidizing isolate. Shown are examples of triplicate cultures inoculated from the same source. An increase in OD is concomitant with the oxidation of VC and ethene. Vertical lines indicate additions of VC or ethene. Grey lines represent abiotic medium controls to account for gas loss during sampling.

6.2 IDENTIFICATION OF THE VC-UTILIZING ORGANISM AND CLONE LIBRARY ANALYSIS

An axenic culture from an isolated colony was designated HF26 and its 16S rRNA gene sequence was determined. Comparison to the NCBI database by BLAST revealed that this sequence was closely related to *Mycobacterium moriokanense* and to a known aerobic VC oxidizer, *Mycobacterium* strain JS619 with BLAST identities of ca. 99% over 1400bp and 450bp, respectively (Coleman et al., 2002) (Figure 6.4). *Mycobacterium* spp. are often found in soils and have been shown to degrade a variety of anthropogenic compounds including VC (Dean-Ross and Cerniglia, 1996; Kamala et al., 1994; Le and Coleman, 2011; Miller et al., 2007).

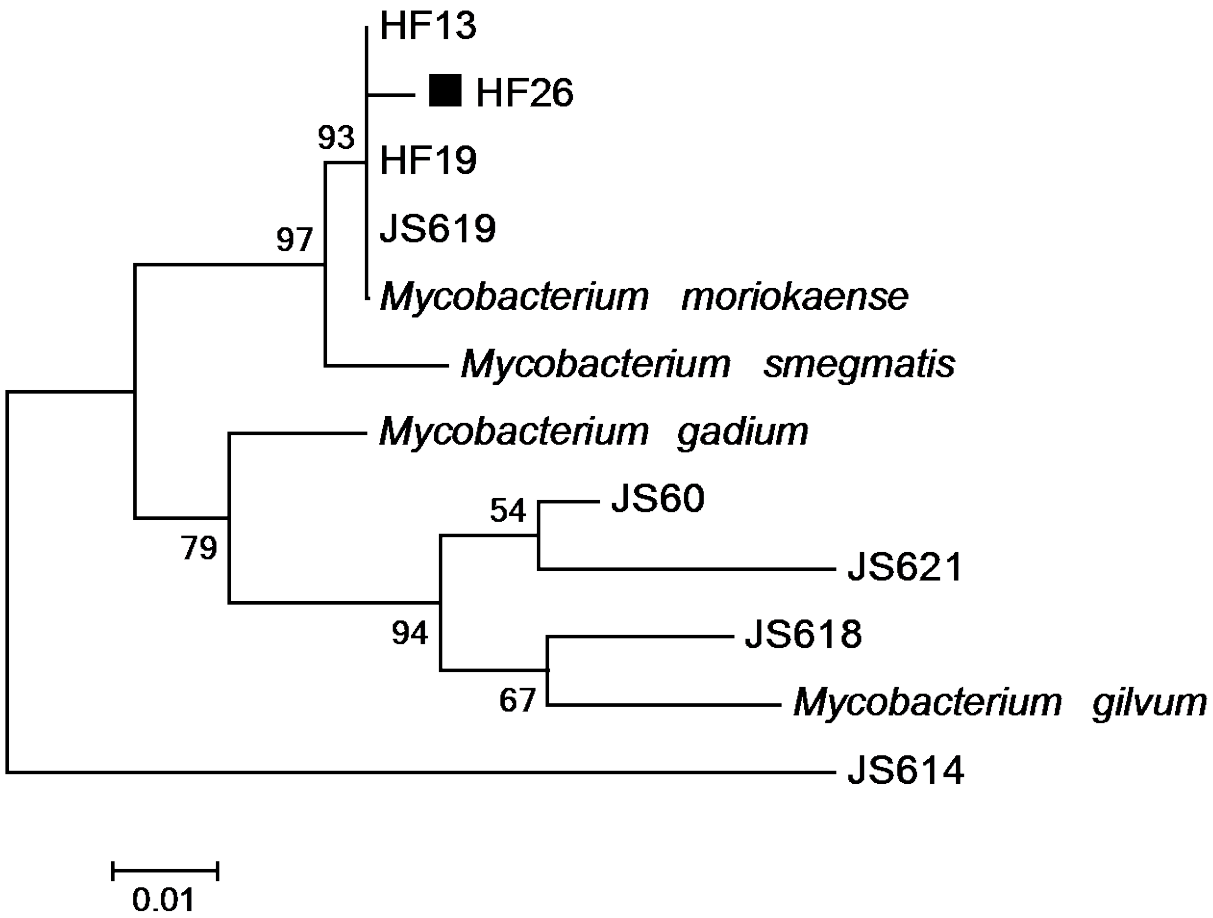


Figure 6.4. 16S rRNA gene tree of aerobic VC oxidizing isolates. Sequence HF26 (■) was obtained from the isolated microaerobic VC oxidizing organism, whereas HF13 and HF19 are sequences from the groundwater microcosm clone library. JS sequences are known VC or ethene oxidizers. Strain JS614, a *Nocardioides* spp., was used as an outgroup in rooting the tree (Coleman et al., 2003).

To determine if the VC oxidation activity observed in the second-generation microcosms was due to organisms similar to this aerobic isolate, a bacterial 16S rRNA gene clone library was constructed with DNA extracted from transfer #2 microcosms and analyzed for restriction fragment length polymorphisms (RFLPs). One or more representatives of unique RFLP patterns were sequenced. As seen in Table 6.1, a RFLP pattern representing 25 out of the 67 clones had sequences nearly identical with *Mycobacterium* strain JS619 and the VC oxidizer isolated in this study (Figure 6.4).

The 16S rRNA gene analysis indicates that the transfer #2 microcosms had infiltration of low levels of oxygen, since the dominant member was an obligate aerobic microorganism. This aerobic VC oxidizing *Mycobacterium* sp. most likely scavenged low levels of oxygen. VC

oxidizers have been found to function down to extremely low levels of oxygen (Bradley, 2000; Gossett, 2010; Mattes et al., 2010). A strict aerobe, *Acinetobacter spp*., colonized biotraps that were sufficiently reduced to also be colonized by sulfate reducers and methanogens present and permit reductive dechlorination of trichlorobenzenes. This aerobe was also shown to play a central role in biodegradation of chlorobenzene (Burns et al., 2013), which reinforces the possibility that strict anaerobes and aerobes may coexist in contaminated groundwater. Likely sources of oxygen in the microcosms and transfers for this study are repetitive syringe sampling for headspace measurements and/or the passage of bottles with multiple septum punctures through the airlock of the anaerobic chamber when returned from sampling; this could have caused a partial vacuum in the vials, pulling in oxygen still present in early evacuation/flush cycles of the airlock. In the subsequent enrichments, butyl rubber stoppers replaced red rubber stoppers, and the vials were not passaged through an airlock and positive pressure was maintained by periodic additions of N_2 to replace gas removed for sampling.

Table 6.2. RFLP pattern analysis of clones from anaerobic VC oxidizing groundwater microcosms.

Best Hit for RFLP	GenBank Accession Number	Percent Identity	Number of RFLPs
Mycobacterium JS619	AY859686.1	98%	25
UC anaerobic actinobacterium[a]	DQ419604.1	94%	10
UC *Desulfobacca*	GU472643.1	97%	9
UC *Bacteroidetes*	JQ580314.1	86%	8
UC *Spirochaetales* bacterium	EU266876.1	99%	7
UC *Chloroflexi* (Not Dhc[b])	HQ183907.1	99%	4
Geobacter pelophilus	U96918.1	98%	4

[a]UC= Uncultured.
[b]Dhc = *Dehalococcoides* spp.

The presence of phylotypes related to known anaerobes (Table 6.1) suggests that conditions were anaerobic in the microcosms at least some of the time. In fact, the resazurin added to these microcosms to indicate oxidation/reduction status remained clear throughout the experiments. Moreover, the presence of potentially anaerobic phylotypes prevents us from conclusively ruling out that all the VC oxidation activity was aerobic, since some of these anaerobes may have participated in VC oxidation.

6.3 CONCLUSIONS

Though initial microcosm results indicated that VC was oxidized under anaerobic conditions, neither enrichment nor isolation of an anaerobic VC oxidizer was successful. All attempts to transfer material from initial microcosms into defined medium or stimulation of anaerobic VC

oxidation activity in these samples failed. The only activity recovered from the groundwater or original microcosms by culturing was the aerobic oxidization of VC. An aerobic VC oxidizer was subsequently isolated and identified as being related to previously described VC oxidizing *Mycobacterium* spp. This phylotype was also the most numerous in a bacterial 16S rRNA gene clone library from the transfer #2 microaerobic microcosms representing 38% of the total clones.

Six years after the original microcosms were established, a fresh set of soil samples and groundwater were obtained from the same industrial site and used to prepare nearly 300 new microcosms. During more than three years of monitoring, the only VC biodegradation activity observed in the microcosms was reduction to ethene. This suggests that conditions at the site had become more reducing and favorable to reductive dechlorination over time.

Despite evidence that anaerobic VC oxidation occurs within contaminated sites, the organisms involved have so far evaded enrichment and identification in the laboratory. At some of these sites, it is possible that oxygen is below the level of detection but high enough to allow for aerobic VC oxidation. Use of Compound-Specific Isotope Analysis can be used to reduce uncertainty about the fate of biotransformed ethenes (Mundle et al., 2012), but cannot fully explain the observed VC loss at contaminated sites. To accurately assess the flux of VC at sites where VC is observed to be decreasing without concomitant ethene formation, detection of known VC-oxidizing phylotypes like *Mycobacterium* spp. within a plume in comparison to non-contaminated groundwater could be indicative of aerobic oxidation of VC at levels of oxygen below detection, as would the ability to enrich aerobic VC oxidizers as we have in this study.

The isolation of an aerobic *Mycobacterium* sp. strain from presumably anaerobic groundwater highlights the fact that groundwater contamination plumes are dynamic, and our understandings of the biogeochemical cycles within are not fully understood. Studies which track VC in shallow aquifers show VC mineralization under hypoxic conditions (Bradley, 2011), though this is the first study to identify an organism that may be responsible for these *in situ* observations. Further studies must be performed to determine whether these strains of *Mycobacterium* spp. play an important role in *in situ* VC mineralization.

7.0 CONCLUSIONS AND IMPLICATIONS

Considerable advances have been made in implementing anaerobic bioremediation of groundwater contaminated with polychlorinated ethenes. Nevertheless, field observations frequently indicate that the amounts of daughter products detected are insufficient to explain the disappearance of the chloroethenes. There are several possible explanations for the lack of mass balances, including: 1) ethene produced from reductive dechlorination is oxidized to CO_2 as soon as it is produced; 2) VC is directly oxidized to CO_2; 3) VC is oxidized to acetate which is further metabolized to CO_2 and CH_4; or 4) oxygen diffuses into the system at below detection limits, and is continually consumed for microaerobic biodegradation of VC.

The results of this project provide definitive evidence in support of the first and fourth explanations. The possibility of ethene oxidation in sulfate reducing zones may explain the lack of mass balances for some plumes. A key outcome of this project was demonstration of anaerobic ethene biodegradation in a sulfate-reducing enrichment culture that use ethene as its sole carbon and energy source. Bradley and Chapelle (2002) previously demonstrated in microcosms the occurrence of ethene bio-oxidation under sulfidogenic conditions. The studies presented here support and expand upon those results. Most significantly, this project yielded a stable enrichment culture that grows via anaerobic oxidation of ethene, based on multiple lines of evidence.

Molecular and microscopic evaluation of the enrichment culture yielded information regarding the microbe most likely responsible for anaerobic ethene bio-oxidation. The most numerous phylotype found in the 16S rRNA gene clone library of the culture, called MT6, is a member of the *Deltaproteobacteria*, most closely related to *Desulfovirga adipica* and several *Syntrophobacter* spp., organisms that carry out reactions with relatively low thermodynamic yields, and is somewhat more distantly related to *Desulfoglaeba alkanexedens*, a hydrocarbon utilizer. The MT6 16S rRNA gene is ≤91% identical with those from cultured organisms, and had ≤93% identity with the entire NCBI nr database, demonstrating how unique this phylotype is. While this distance precludes physiological conclusions based on phylotype, it is a reasonable candidate for an organism responsible for ethene oxidation and sulfate reduction. The dominant morphotype in the cultures was a relatively large egg shaped cell, and it is tempting to conclude that the MT6 phylotype has this morphology which many organisms with related sequences share, but further studies are necessary to demonstrate this proposition.

The availability of a stable enrichment culture opens up opportunities for developing tools to evaluate the occurrence of anaerobic ethene bio-oxidation. For example, it will now be possible to evaluate the stable isotope fractionation signature of this process, as has been done for ethene reduction to ethane (Mundle et al., 2012). However, when ethene is reduced to ethane *in situ*, the ethane can be readily detected by gas chromatography and contribute to mass balance determinations. If ethene is oxidized, it joins the large CO_2 pool and cannot be accounted for in the mass balance, leading to a deficit. Presently, there is no way to predict whether this reaction is occurring other than microcosm studies. It is possible that anaerobic ethene oxidation will have a stable isotope fractionation signature. It is also possible that the unique bacterial phylotype we have found associated with this reaction can serve as a biomarker for it, but considerably more study will be needed to support or refute this hypothesis.

With respect to the fourth explanation, another key outcome of this project was isolation of a *Mycobacterium* sp. that grows under microaerobic conditions with VC as its sole source of carbon and energy. Several previous studies have reported isolation of mycobacteria and other microbes that aerobically biodegrade VC. These efforts were consistently initiated under aerobic conditions. A key outcome of our work was identification of an aerobic VC degrader from presumably anaerobic microcosms. The results are consistent with other studies that indicate aerobic VC oxidizers have a very high affinity for oxygen (Coleman et al., 2002) and are capable of biodegrading VC at oxygen levels that are below detection (Gossett, 2010). The role of aerobic biodegradation at very low oxygen concentrations needs further investigation at field sites to determine the contribution of this process to incomplete mass balances of chlorinated ethene daughter products.

The isolation of an aerobic *Mycobacterium* sp. strain from presumably anaerobic groundwater highlights the fact that groundwater contamination plumes are dynamic, and our understanding of the biogeochemical cycles within are not fully developed. Studies which track VC in shallow aquifers show VC mineralization under hypoxic conditions (Bradley, 2011), though our work is the first to identify an organism that may be responsible for these *in situ* observations. Further studies must be performed to determine whether these strains of *Mycobacterium* spp. play an important role in *in situ* VC mineralization. For example, detection of known VC-oxidizing phylotypes like *Mycobacterium* spp. within a plume in comparison to non-contaminated groundwater could be indicative of aerobic oxidation of VC at levels of oxygen below detection, as would the ability to enrich aerobic VC oxidizers as we have in this study.

This project included preparation of more than 700 microcosms with soil and groundwater samples from 11 sites. Nevertheless, definitive evidence in support of anaerobic bio-oxidation of VC was not found. In a majority of the several hundred microcosms that were monitored (usually for one year or longer), VC was either recalcitrant or underwent reductive dechlorination to ethene. Consumption of VC without accumulation of ethene or ethane did occur in microcosms from one location at one of the sites. However, attempts to link the biodegradation of VC to a specific anaerobic electron acceptor were unsuccessful, as were attempts to enrich for VC biodegradation by transfers to groundwater or medium. Furthermore, the possibility of oxygen serving as the electron acceptor (via diffusion through the septa) could not be completely ruled out. The sulfate-reducing enrichment culture described above that grows on ethene was unable to biodegrade VC. Consequently, a key need of SON Number ERSON-07-04 remains unfulfilled, i.e., identification of microbes capable of growing on VC via anaerobic bio-oxidation. Given the lack of success in finding such microbes after such a widespread search, further efforts towards this goal are likely to be high risk. Nevertheless, it remains a worthwhile venture, since it seems unlikely that anaerobic ethene oxidation under sulfidogenic conditions and microaerobic VC biodegradation can explain all of the incomplete mass balances for chlorinated ethenes observed in the field.

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APPENDIX A: SUPPORTING DATA

A-1: SUPPORTING DATA FOR SECTION 4.0

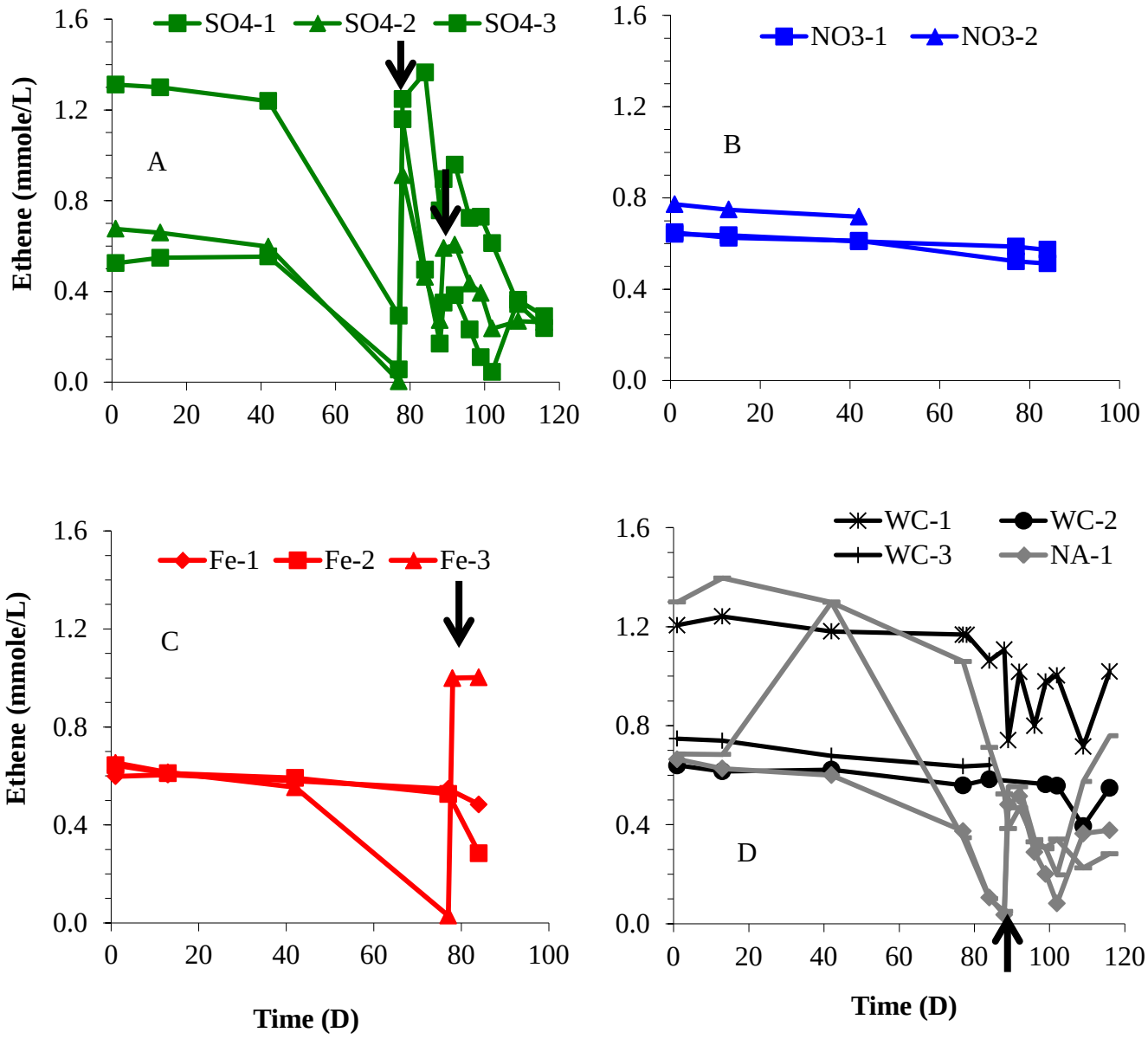
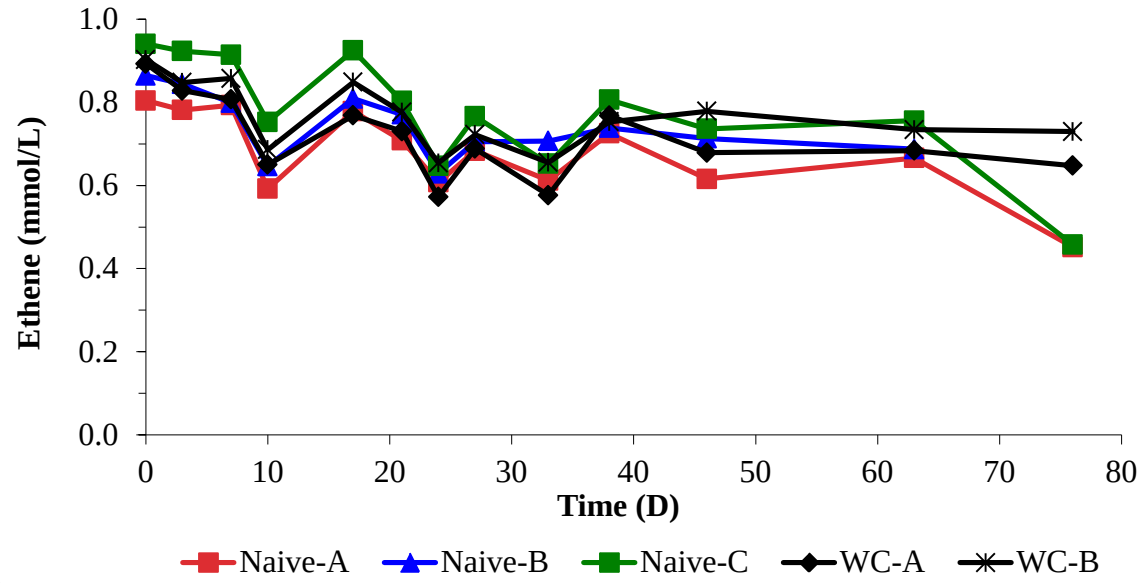


Figure A-1. Salem Canal sediment microcosms amended with (A) 5 mM sodium sulfate, (B) 5 mM sodium nitrate, (C) 5 mM amorphous iron III hydroxide or (D) no addition (NA). WC, water control. Arrows represent an addition of ethene.

A



B

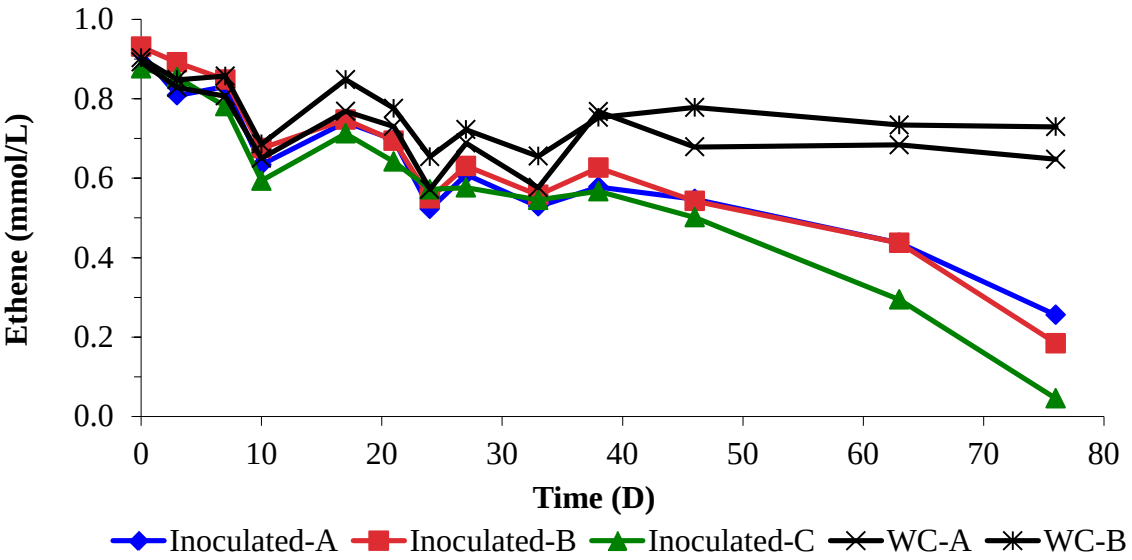


Figure A-2. Salem Canal sediment microcosms the received (A) only sediment and anaerobic deionized water (i.e., "naïve") or (B) sediment and anaerobic deionized water plus 1% inoculum from other active microcosms (i.e., "inoculated"). WC, water control.

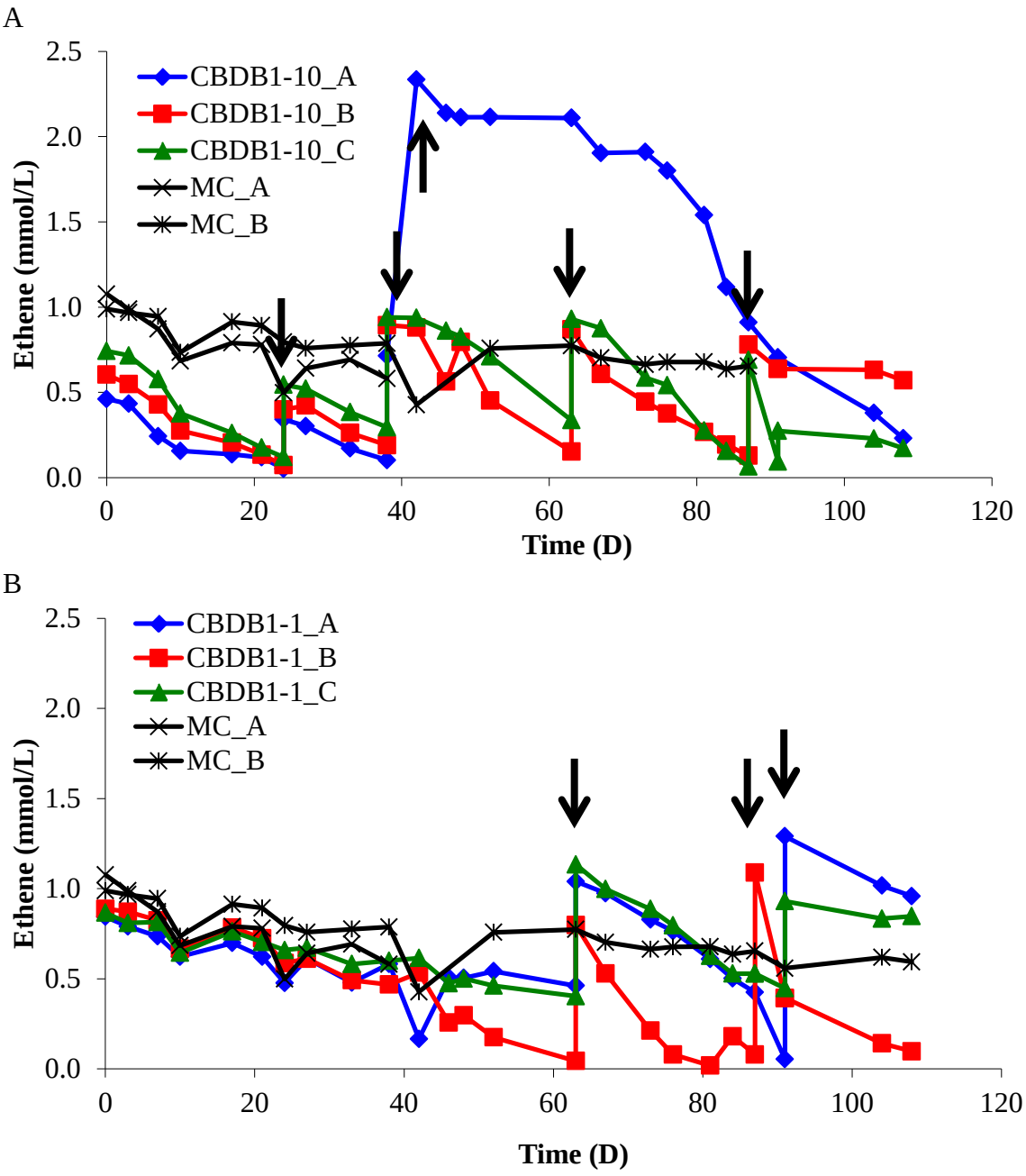
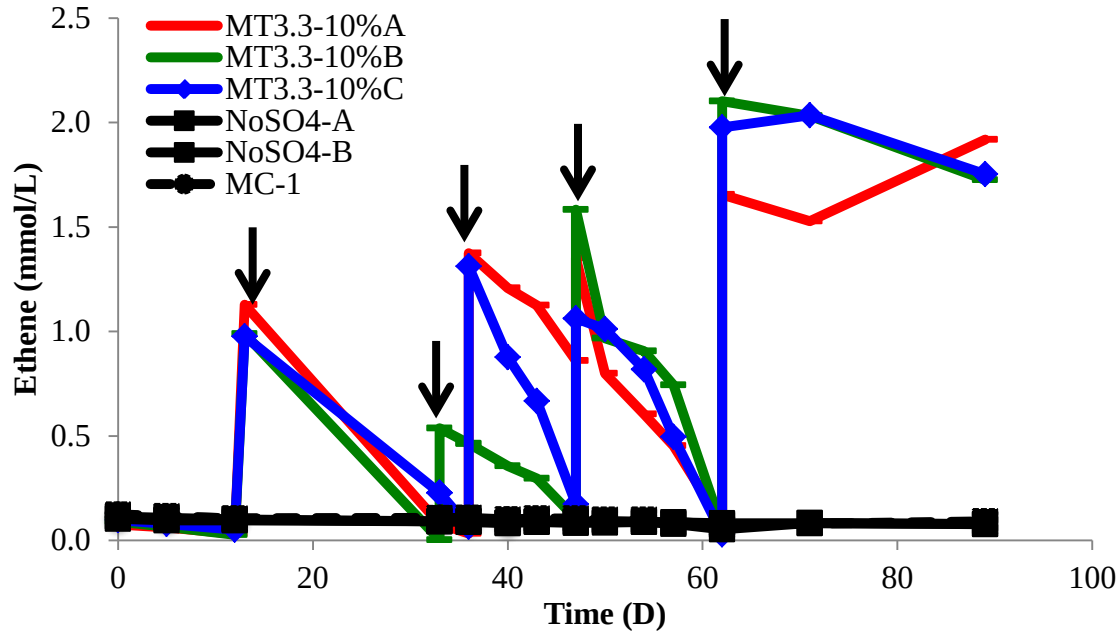


Figure A-3. First generation enrichment cultures, inoculated at (A) 10% or (B) 1%, with MC serving as the abiotic medium control. Both treatments inoculated from the same microcosm. Arrows represent an addition of ethene.

A



B

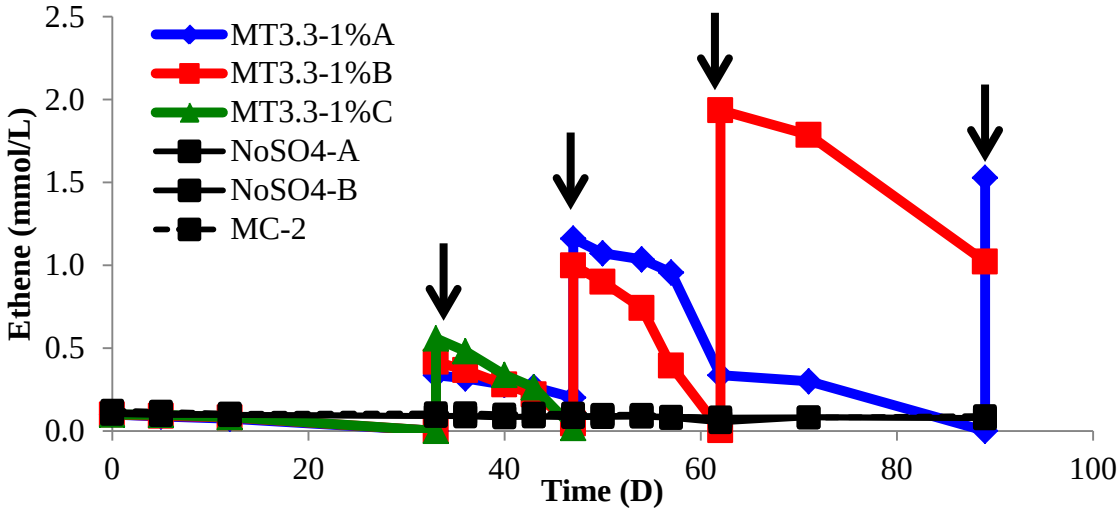


Figure A-4. Third generation enrichment cultures, inoculated at (A) 10% or (B) 1%, with MC serving as the abiotic medium control. "$NoSO_4$" bottles were inoculated at 1%. All treatments inoculated from the same second generation enrichment culture. Arrows represent an addition of ethene.

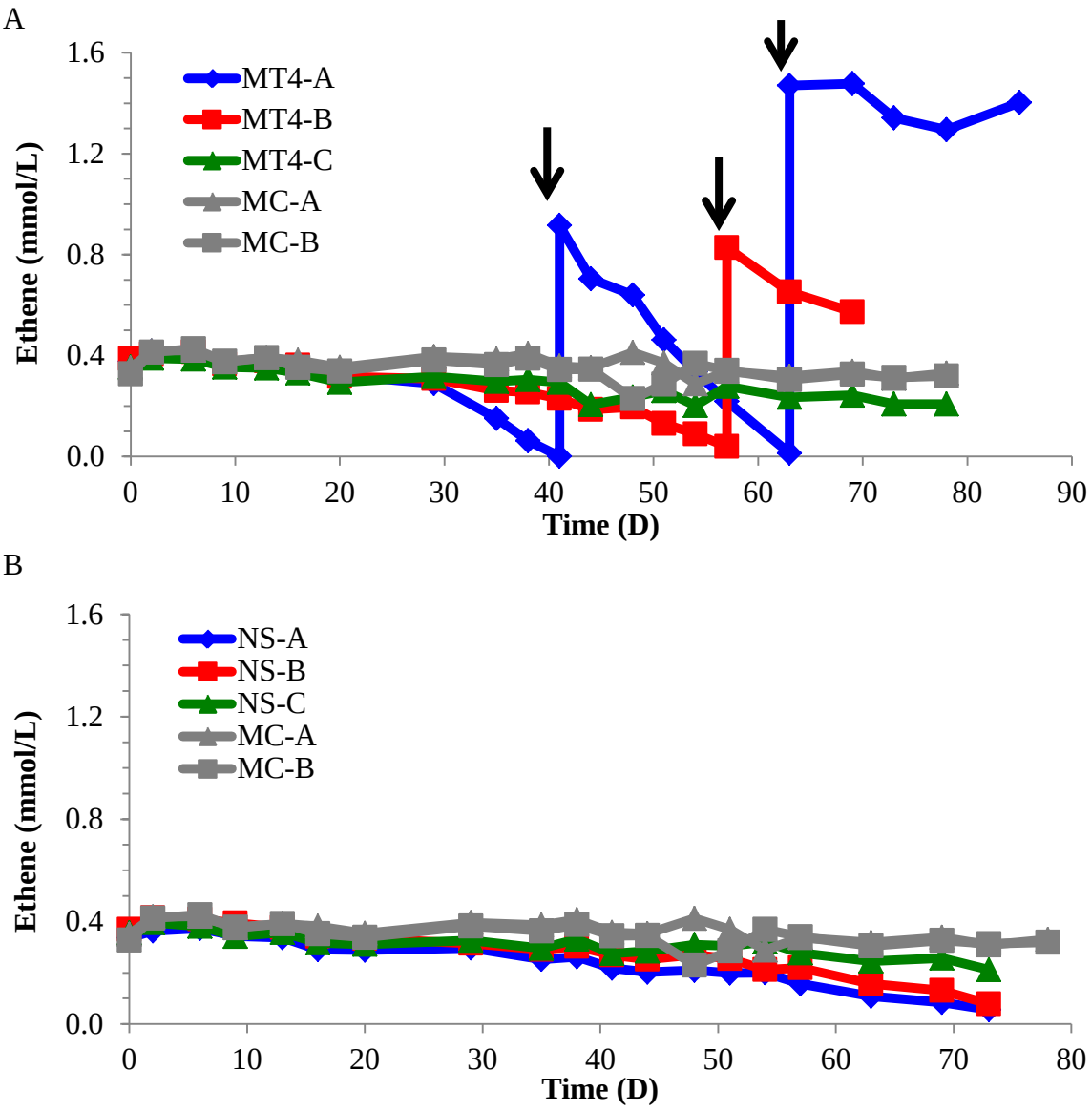
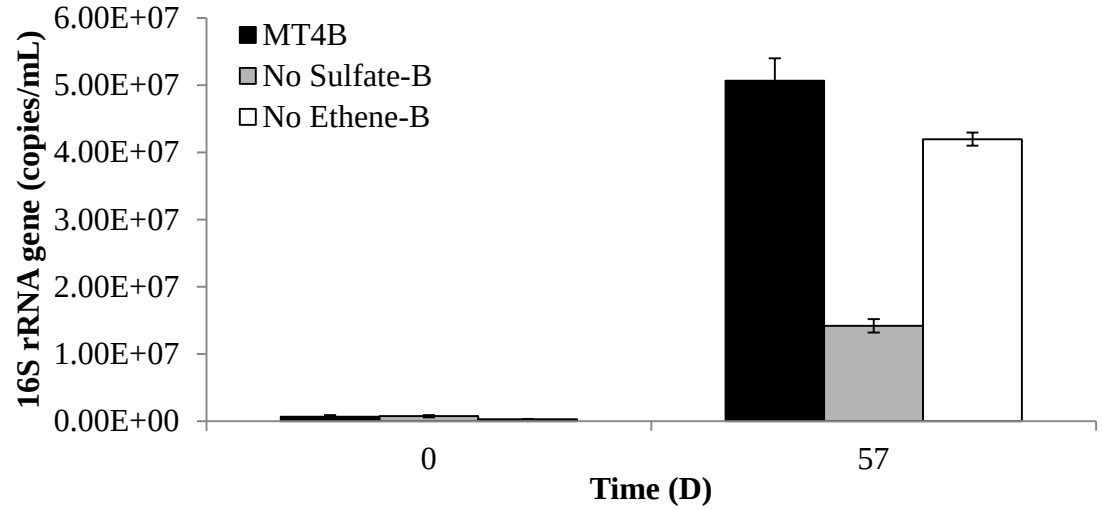


Figure A-5. Fourth generation enrichment cultures, inoculated at 2% (A) with sulfate or (B) without sulfate, with MC serving as the abiotic medium control. All treatments inoculated from the same third generation enrichment culture. Arrows represent an addition of ethene. After 78 days MT4 failed to show a decrease in ethene concentration, which could be a result of the low inoculum as shown by qPCR on MT4A and MT4B.

A



B

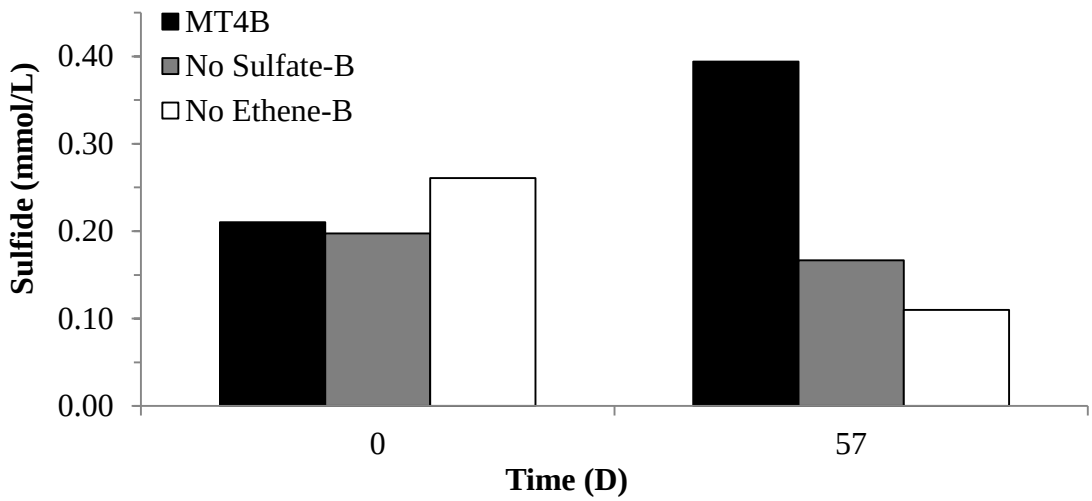


Figure A-6. (A) Increase in 16S rRNA gene copies and (B) production of sulfide from a fourth generation enrichment culture that consumed the first 0.4 mM ethene after 57 days. The no sulfate and no ethene controls increased in 16S rRNA copy number but not to the same extent as MT4B. They also showed a slight decrease in sulfide concentration from day zero. The sulfide:ethene molar ratio for this culture is 1.14. On day 69, this culture was lost (broken).

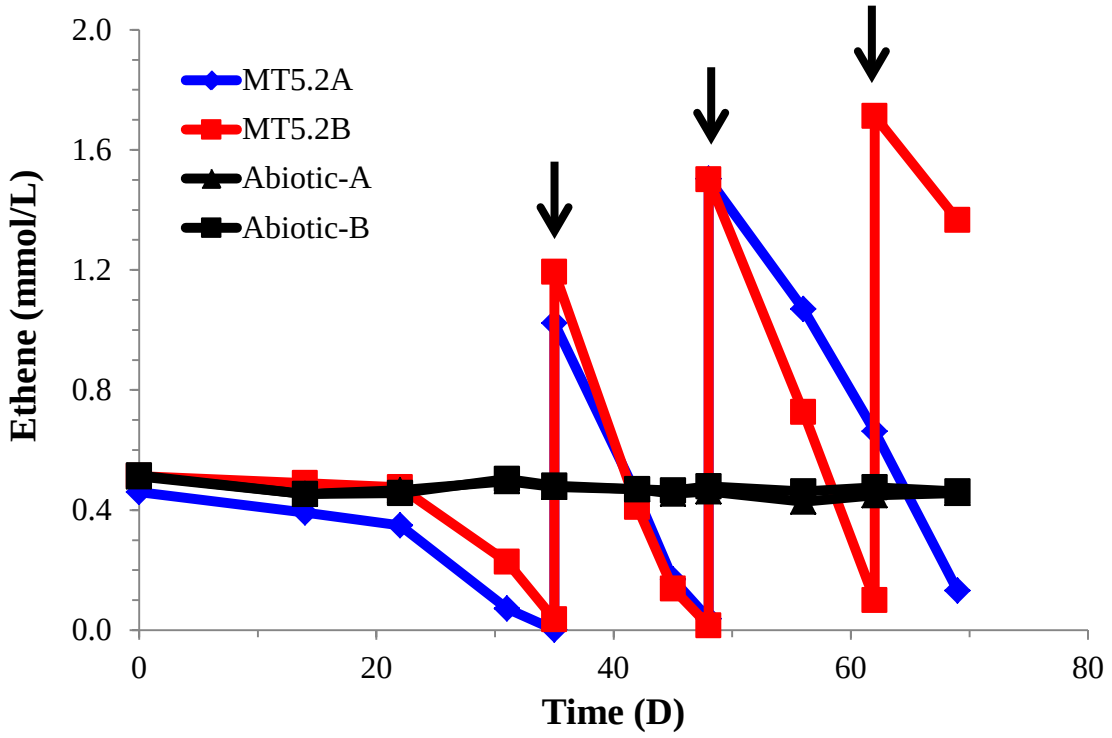


Figure A-7. Fifth generation enrichment cultures, inoculated at 1%. Both treatments were inoculated from the same fourth generation enrichment culture. Arrows represent an addition of ethene.

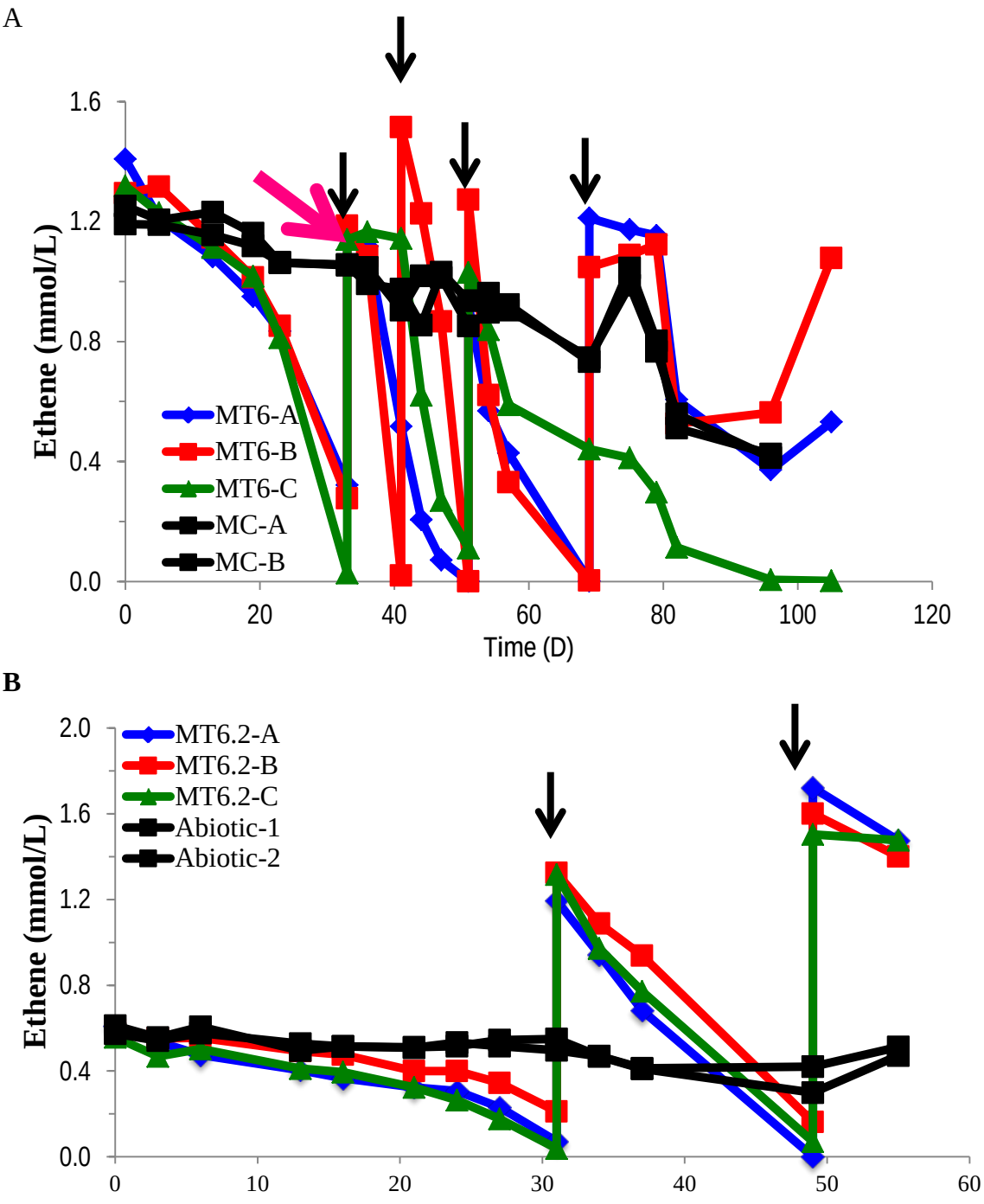


Figure A-8. Sixth generation enrichment cultures (A) that did not receive [^{14}C]ethene and (B) that did receive [^{14}C]ethene. Cultures in (A) and (B) were inoculated at 1% from MT5.2-B. Black arrows represent an addition of ethene. Pink arrow represents when DNA was extracted for clone library analysis.

A-2: SUPPORTING DATA FOR SECTION 6.0

RESULTS FOR THE INITIAL MICROCOSM EXHIBITING VC BIODEGRADATION

Section 6.1 mentions results for the first set of microcosms. These included a treatment that received only soil and groundwater (designated "as-is"). Triplicate bottles were prepared. The main contaminants present were trichloroethene (TCE), *cis*-dichloroethene (cDCE), dichloromethane (DCM), and vinyl chloride (VC). Results for one of the bottles (L1-E#1) are shown in Figure A-9. On day 232, [^{14}C]VC was added to the bottles, which explains the jump in concentration on this day. The next time the bottles were monitored (day 263), the concentration of VC rose further, presumably since the microcosms were not at equilibrium when they were monitored on day 232. By day 305, most of the VC was consumed in bottle L1-E#1; however, a low level (ca. 0.2 µM) persisted through day 390.

The other two microcosms (bottles L1-E#2 and #3) behaved differently. After adding [^{14}C]VC on day 232, the concentration of VC did not decrease appreciably through day 390. The fate of the [^{14}C]VC in the three microcosms was evaluated on day 390 (Figure A-10). In bottle L1-E#1 (with presumptive anaerobic bio-oxidation of VC; Figure B-1), approximately 30% of the radiolabel was recovered at $^{14}CO_2$. A significant amount of the ^{14}C activity was unaccounted for, apparently due to partitioning of carbonates into the soil, which was not assayed. In the two microcosms that did not exhibit any biodegradation of VC (bottles L1-E#2 and #3), the main ^{14}C activity recovered was VC.

In the three microcosms, the concentration of TCE and cDCE decreased gradually, as shown in Figure A-9 for bottle L1-E#1. However, compared to the autoclaved controls (AC), the magnitude of decrease was equivalent or lower. Figure A-11 shows the average percent decrease in TCE and cDCE for the triplicate "as-is" microcosms (designated "Live") compared to the autoclaved controls.

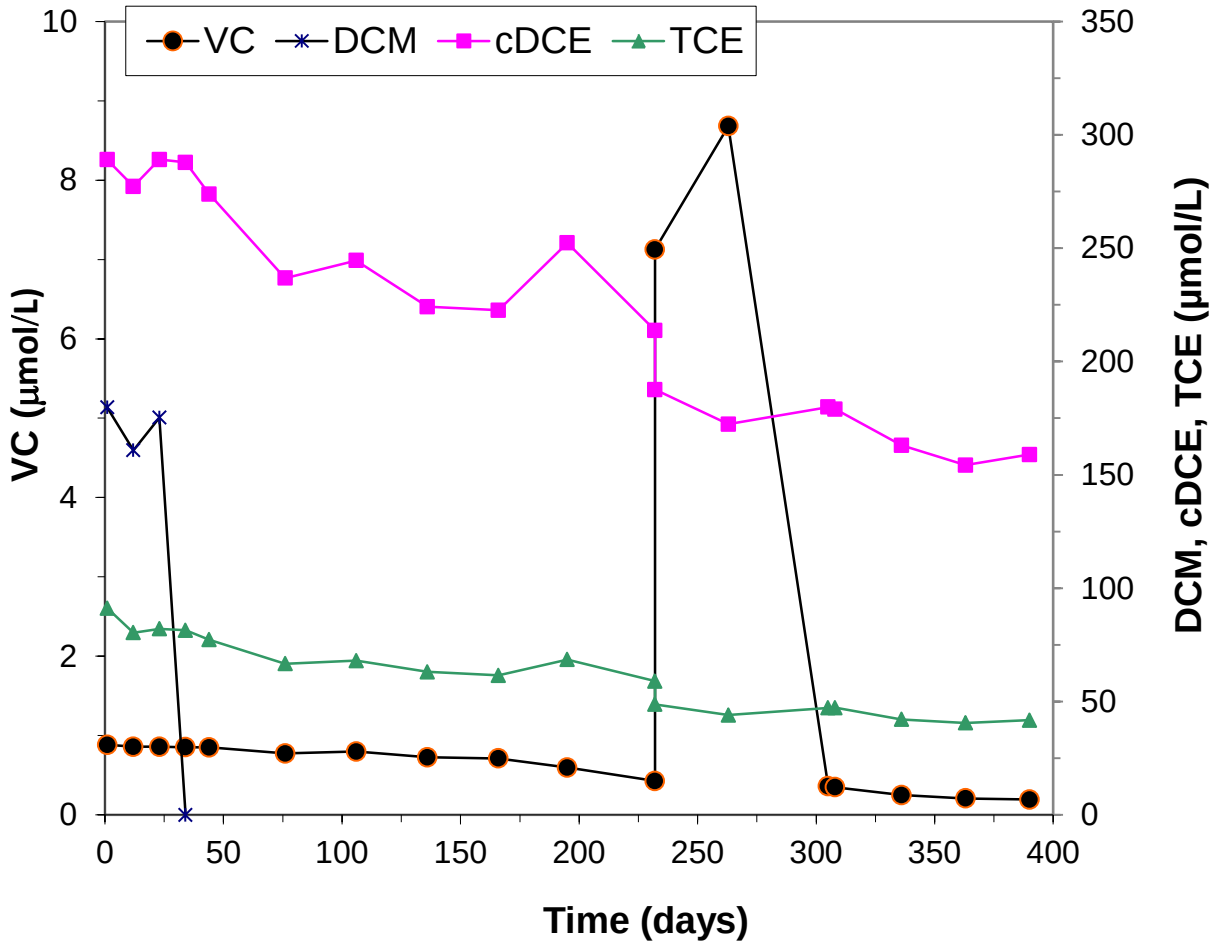


Figure A-9. Results for the as-is microcosm, L1-E#1. The increase in VC concentration on day 232 was a consequence of adding [^{14}C]VC.

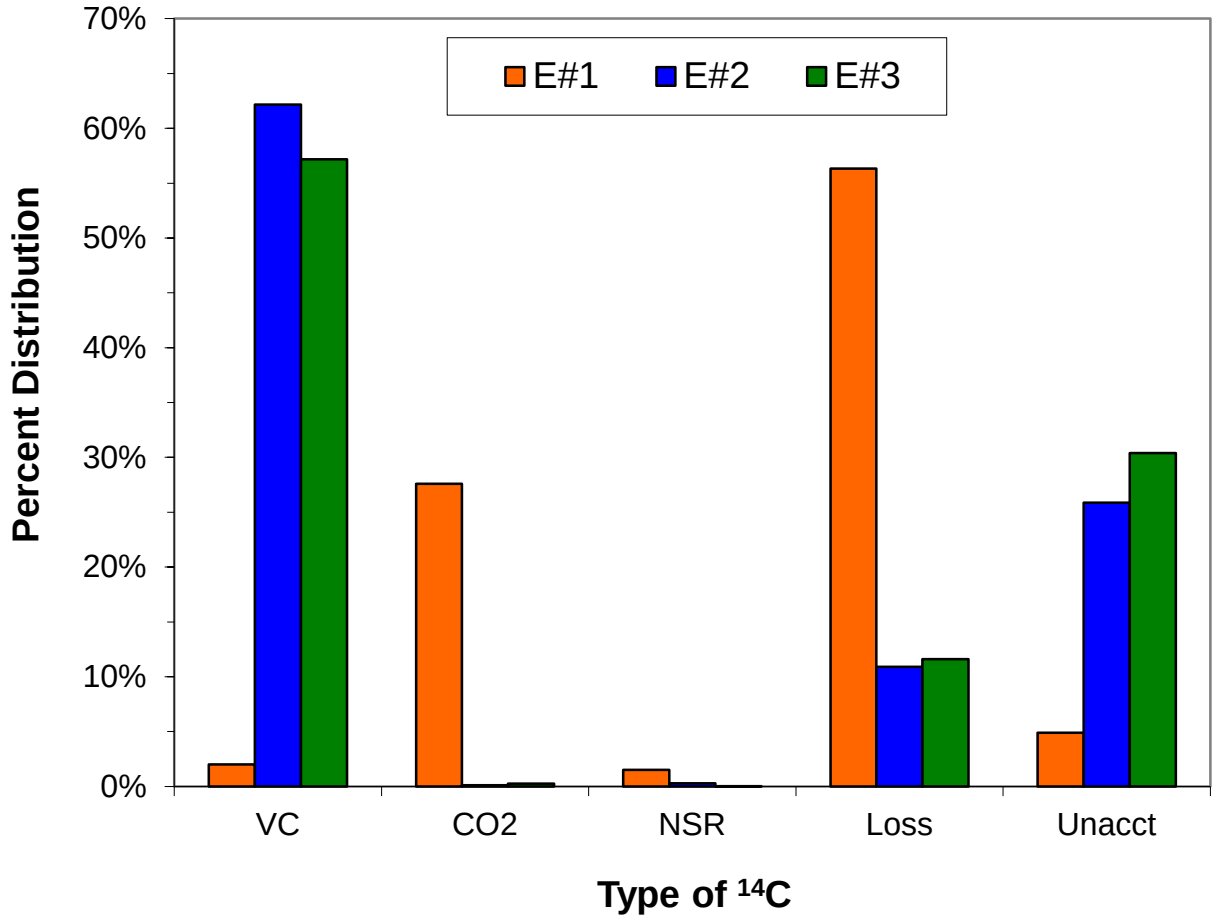


Figure A-10. Distribution of ^{14}C in the "as-is" microcosms (bottles L1-E#1, #2 and #3). NSR = non-strippable residue (activity remaining after stripping at pH ca. 3); Unacct = unaccounted for (i.e., ^{14}C that was present but could not be assigned to VC, CO_2 or NSR). Loss is the difference between ^{14}C measured when [^{14}C]VC was first added (day 232) and the total ^{14}C remaining when the bottles were sacrificed (day 390).

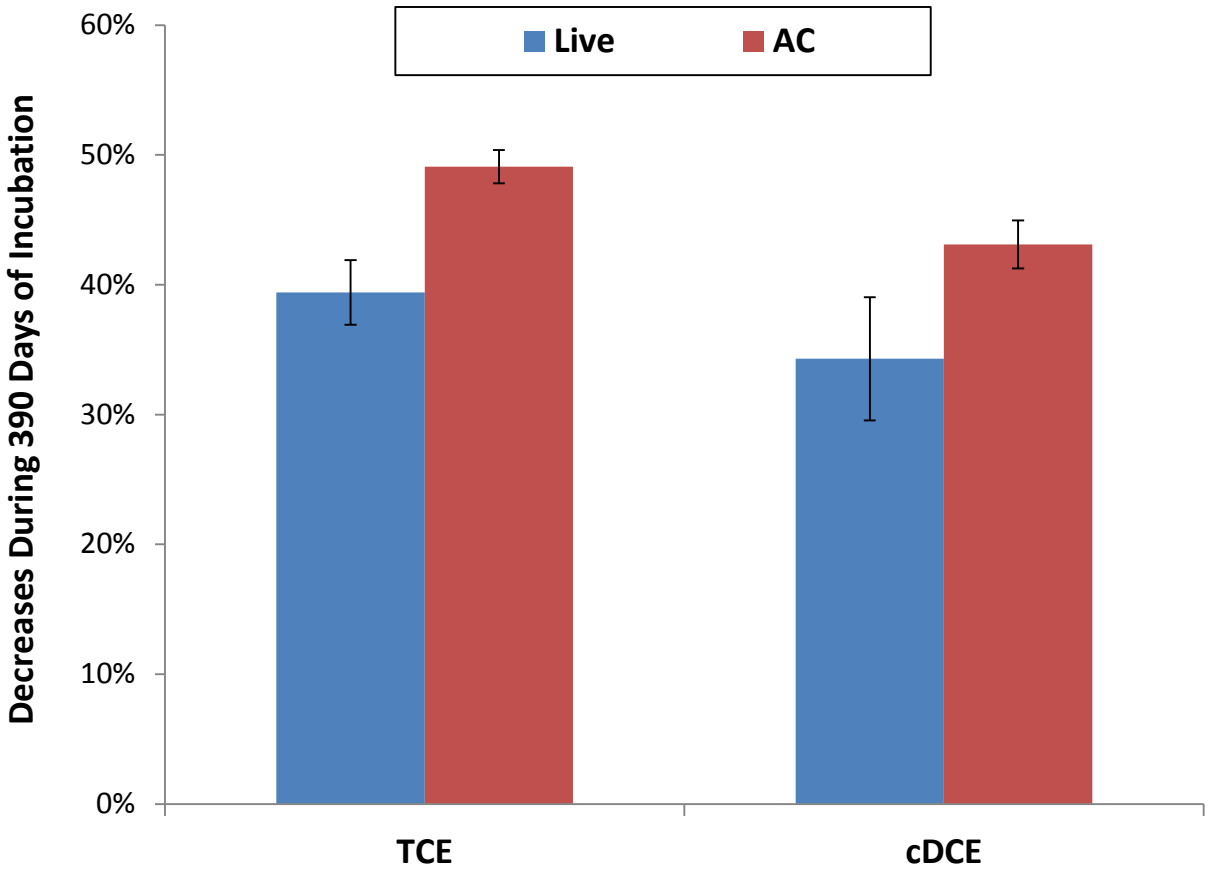


Figure A-11. Average decreases for TCE and cDCE in the "as-is" microcosms (bottles L1-E#1, #2 and #3, designated "Live") compared to the autoclaved controls (AC). Error bars represent the standard deviation for triplicate bottles.

APPENDIX B: LIST OF SCIENTIFIC/TECHNICAL PRESENTATIONS

Articles in Peer-Reviewed Journals

Fullerton, H., Crawford, M., Bakenne, A., Freedman, D. L. and Zinder, S. H. Anaerobic oxidation of ethene coupled to sulfate reduction in microcosms and enrichment cultures. *Environ. Sci. Technol.*, **accepted** (2013).

Fullerton, H., Rodgers, R., Freedman, D. L. and Zinder, S. H. Isolation of an aerobic vinyl chloride oxidizer from anaerobic groundwater." *Chemosphere*, **in review** (2013).

Posters

Freedman, D. L., High, J., Lehmicke, L., and Zinder, S. H. Characterization of Microbes Capable of Using Vinyl Chloride as a Sole Carbon and Energy Source by Anaerobic Oxidation. SERDP and ESTCP Technical Symposium, Washington, DC (December 4-6, 2007).

Freedman, D. L., High, J., Reid, A., Fullerton, H., Lehmicke, L., and Zinder, S. H. Searching for Elusive Microbes that Anaerobically Oxidize Vinyl Chloride. SERDP and ESTCP Technical Symposium, Washington, DC (December 2-4, 2008).

Fullerton, H., High, J., Reid, A., Lehmicke, L., Freedman, D. L. and Zinder, S. H. Isolation of Aerobic Vinyl Chloride Oxidizing Microorganisms from Anaerobic Groundwater Microcosms. *Abstracts from the 109th General Meeting of the American Society of Microbiology*, Philadelphia, PA (May 17-21, 2009).

Freedman, D. L., Reid, A., Fullerton, H., and Zinder, S. H. Observations of Ethene and Vinyl Chloride Bio-Oxidation Under Anaerobic Conditions." SERDP and ESTCP Technical Symposium, Washington, DC (December 1-3, 2009).

Fullerton, H. and Zinder, S. H. Anaerobic Oxidation of Ethene Coupled to Sulfate Reduction. *Abstracts from the 112th General Meeting of the American Society of Microbiology*, San Francisco, CA (June, 2012).

Theses

High, J. M., Microcosm Evaluation of Vinyl Chloride Bio-Oxidation Under Anaerobic Conditions. M.S. Thesis, Clemson University (August 2008).

Reid, A., Anaerobic Bio-oxidation of Vinyl Chloride and Ethene. M.S. Thesis, Clemson University (August 2010).

Bakenne, A. Assessing Anaerobic Bio-Oxidation of Vinyl Chloride and Ethene in Microcosms. M.S. Thesis, Clemson University (August 2012).

Fullerton, H. Growth on Anthropogenic Compounds by Reductive Dehalogenation and Oxidation. Ph.D. Dissertation, Cornell University (2012).